THE HPLC SOLVENT GUIDE

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NITRILES AND NITROGENOUS SOLVENTS

By far the most commonly used organic mobile-phase component is acetonitrile. It offers a unique set of properties which set it apart from the other LC solvents:

1. It is moderately polar.
2. It has a midrange solvent strength.
3. It is, overall, an excellent solubilizing solvent and typically generates sharp well-defined chromatographic peaks.
4. It is miscible with a wide range of organic solvents as well as water, and its mixtures with water are of low viscosity when compared with analogous alcohol or ether mixtures.
5. It is a weak hydrogen bond acceptor.
6. It has a very low UV cutoff.

The areas of widest application for acetonitrile include pharmaceutical, amino acid, peptide and protein, and various biological compound analyses. Acetonitrile has found limited use in the analysis of polymers.

It should be noted, especially in light of all the positive properties that acetonitrile offers for use in LC, that acetonitrile presents at least two drawbacks: (1) phosphate buffers, especially under multiply charged buffer pH conditions, have very limited solubility in acetonitrile; and (2) acetonitrile is fairly unstable and reactive with strong acids (e.g., sulfuric acid).

Propionitrile and butyronitrile have been used in very limited and specific cases—namely, in the analysis of lipids where acetonitrile does not effectively solubilize the sample. The major drawbacks to the use of these solvents are the inability to pur-

chase them in as high a purity as acetonitrile and the increased health hazards associated with their use as compared with acetonitrile.

Most of the nitrogenous compounds used as solvents in HPLC are polar strong hydrogen-bonding species. The most common are triethylamine (TEA) and diethylamine. Pyridine may be included in this group; however, its high UV cutoff (330 nm) and pungent odor severely limits its utility. Some of the major advantages to their use are

1. They have reasonably low UV cutoffs (not pyridine).
2. They are very volatile so that a sample can be recovered easily.
3. They are strong hydrogen-bonding compounds, making them ideal for use as silanol group blocking agents.
4. They are soluble in most solvent systems at a useful level ($< 2\%$).

TEA and diethylamine are very strong bases. Therefore, they will aggressively dissolve silica-based support materials. In order to protect the analytical column from a rapid and irreversible "death," a precolumn should be used. A precolumn, which is positioned after the pump head and before the injector, contains packing material that matches the analytical column in functionality (e.g., octyl, octadecyl, underivatized base silica) but can be made from large particle (40–60 mesh) material. These columns can be effectively and efficiently dry-packed in the laboratory and should be regenerated (i.e., the packing material should be completely replaced) frequently.

Anyone who has worked with these amine compounds will immediately be able to relate a major drawback to their use. They are noxiously odiferous (i.e., they stink). Their characteristic "fishlike" odor, once experienced, is quite unforgettable.

Dimethylacetamide and dimethylformamide are extremely polar hydrogen-bond-accepting compounds. They are such strong solvents that they have found little use in HPLC. They do offer unique selectivity properties and therefore should at least be kept in mind when developing complex separations of highly polar compounds.

7.1 IMPURITIES

Acetonitrile is typically a by-product of the large-scale production of acrylonitrile (from ammonia and propylene) and can contain a wide range of very low level impurities (785). These include acrylonitrile, allyl alcohol, acrylic acid, and acetic acid. Because of the widespread use of acetonitrile in many synthetic and chromatographic uses, much attention has been given to the development of purification methods, which essentially eliminates the presence of these impurities. Since the vast majority of acetonitrile use in LC is in reversed-phase separations where almost invariably one of the components is water, one "impurity" that is of little or no concern is water. The reason for mentioning this at all is that most manufacturers now offer

a low-water-content acetonitrile (< 10 ppm water as compared with 30–100 ppm typical) which is intended for use in water-sensitive biosynthetic work. Purchase of this premium-cost material is rarely warranted for RP work.

Propionitrile and butyronitrile contain all the alcohol and acid impurities of acetonitrile plus their isomeric and unsaturated analogs. They are not readily available at purities of > 97%. These solvents are expensive when compared with acetonitrile.

The impurities diethylamine and triethylamine typically contain are propyl and methyl substituents in place of an ethyl group. Depending on the method of production and purification, oxidized forms may also be present. The presence of inorganic acids can produce salt formation.

Pyridine is typically a synthetic that can be produced from a number of different starting compounds (e.g., crotonaldehyde, ammonia, water, and formaldehyde) (786). One advantage of pyridine is that it is difficult to oxidize. Nevertheless, pyridine usually contains the methylated analogs (i.e., lutidines and picolines) and other saturated cyclic nitrogen-containing compounds.

Pure dimethylformamide is colorless and odorless. It slowly hydrolyzes to formic acid and dimethyl amine when in contact with water (787).

Tables 7.1–7.4 list some important chemical, physical, and chromatographic properties as well as general manufacturing and safety parameters for the nitriles and nitrogenous solvents (67–75). Figure 7.1 shows the structure of the solvents listed in Tables 7.1–7.4.

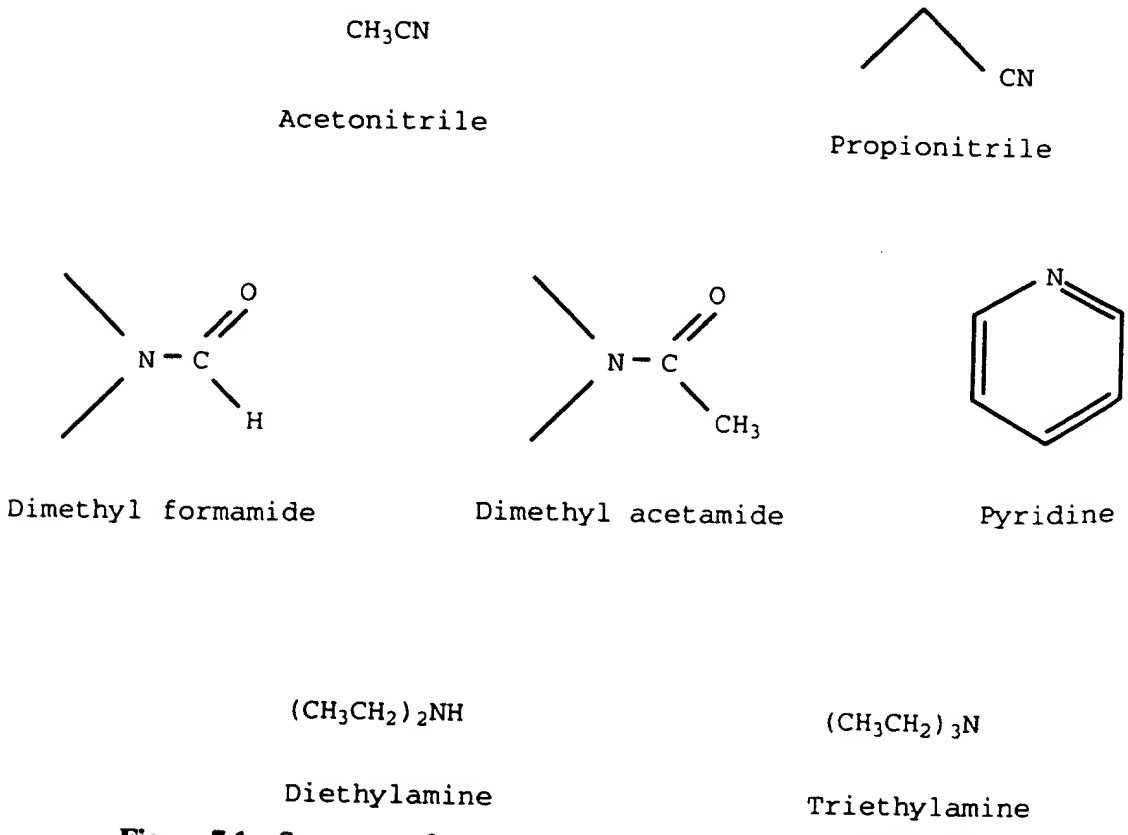


Figure 7.1 Structures of common LC nitriles and nitrogenous solvents.

TABLE 7.1. Physical Properties of Nitriles and Nitrogenous Solvents[a]

	ACN	PRN	DMF	DMA	DEA	TEA	Pyr
Molecular weight	41.05	55.08	73.10	87.12	73.14	101.19	79.10
Density (g/mL)	0.7822	0.7774	0.9487	0.9366[b]	0.707	0.726	0.9832
Viscosity (cP)	0.38[c]	0.29[b]	0.92	0.838[b]	—	—	0.95
Solubility in water (%)	100	—	100	100	100	100	100
Water solubility in solvent (%)	100	—	100	100	100	100	100
Boiling point (°C)	81.60	97.4	153.0	166.1	55	88.8	115.25
Melting point (°C)	−43.8	−92.8	−60.4	−20	−50	−115	−41.55
Refractive index (n_D)	1.3441	1.3655	1.4305	1.4384	1.3861	1.4000	1.5102
Dielectric constant	37.5	29.7	36.71[b]	37.78[b]	—	—	12.4
Dipole moment	3.44	4.05	3.86[b]	3.72[b]	—	—	2.37[b]
Surface tension (dyn/cm)	19.10	26.75[b]	36.76	32.43[d]	—	—	36.88

[a]All values (except boiling and freezing points) at 20°C unless otherwise noted. *Abbreviations*: ACN, acetonitrile, methyl cyanide; PRN, propionitrile; DMF, dimethylformamide; DMA, dimethylacetamide; DEA, diethylamine; TEA, triethylamine; Pyr, pyridine.
[b]At 25°C.
[c]At 15°C.
[d]At 30°C.

TABLE 7.2. Chromatographic Parameters of Nitriles and Nitrogenous Solvents[a]

	ACN	PRN	DMF	DMA	Pyr
Eluotropic strength ($\varepsilon°$) on Al_2O_3	0.65	—	—	—	0.71
Eluotropic strength ($\varepsilon°$) on SiOH	0.52	—	—	—	—
Eluotropic strength ($\varepsilon°$) on C_{18}	3.1	—	7.6	—	—
Solvent-strength parameter, P'	—	—	—	—	—
Hildebrandt solubility parameter, δ	11.9	10.7	11.8	10.8	10.6
Hydrogen-bond acidity, α	0.19	—	0.00	0.00	0.00
Hydrogen-bond basicity, β	0.31	0.37	0.69	0.76	0.64
Dipolarity/polarizability, π^*	0.75	0.71	0.88	0.88	0.87

[a]*Abbreviations*: ACN, acetonitrile, methyl cyanide; PRN, propionitrile; DMF, dimethylformamide; DMA, dimethylacetamide; Pyr, pyridine.

TABLE 7.3. Common Manufacturing Quality Specifications of Nitriles and Nitrogenous Solvents[a]

	ACN	PRN	DMF	DMA	DEA	TEA	Pyr
UV cutoff (nm)	190	—	268	268	—	—	330
Percent water (maximum)	0.01	—	0.03	0.03	—	—	0.01
Available as ACS-tested[b]	ABEFJM	NA[c]	AFJM	—	NA	NA	ABJM
Available as HPLC-grade[b]	ABEFJM	—	ABEJM	ABEM	—	BF	ABEJM
Available through[d]	—	AE	—	—	AEFJM	AJEM	—

[a]*Abbreviations*: ACN, acetonitrile, methyl cyanide; PRN, propionitrile; DMF, dimethylformamide; DMA, dimethylacetamide; Pyr, pyridine.
[b]Manufacturer code: A = Aldrich; B = Burdick & Jackson; E = EM Science; F = Fisher; J = JT Baker; M = Mallinckrodt.
[c]No ACS test exists for this solvent.
[d]Available as a high-purity solvent but not specifically designated as ACS- or HPLC-grade. This does *not* mean a lesser-quality solvent, just that it is not specifically tested for these applications. Manufacturers who produce either ACS- or HPLC-grade solvent, are not listed under this heading.

TABLE 7.4 Safety Parameters of Nitriles and Nitrogenous Solvents[a]

	ACN	PRN	DMF	DMA	DEA	TEA	Pyr
Flash point[b] (TCC)(°C)	5.6	36	58	63	–28	6	68
Vapor pressure (torr at 20°C)	88.8[c]	47.22-[c]	2.7	1.3-[c]	—	54	20.73[c]
Threshold limit value (ppm)	—	—	10	10	—	—	—
CAS number	[*75-05-8*]	[*107-12-0*]	[*68-12-2*]	[*127-19-5*]	[*109-89-7*]	[*121-44-8*]	[*110-86-1*]
Flammability[d]	3	3	2	2	3	3	3
Reactivity[d]	0	1	0	0	0	0	0
Health[d]	2	4	1	2	3	3	2

[a]Abbreviations: ACN, acetonitrile, methyl cyanide; PRN, propionitrile; DMF, -dimethylformamide; DMA, dimethylacetamide; DEA, diethylamine; TEA, triethylamine, Pyr, pyridine.

[b]TCC=TAG closed cup.

[c]At 25°C.

[d]According to National Fire Protection Association ratings (75):

Fire:
- 4 = Materials that vaporize at room temperature and pressure and burn readily.
- 3 = Liquids or solids that can ignite under room conditions.
- 2 = Materials that ignite with elevated temperature or with moderate heat.
- 1 = Materials that must be preheated before they ignite.
- 0 = Materials that will not burn.

Reactivity:
- 4 = Materials that, by themselves, can detonate or explode under room conditions.
- 3 = Materials that can detonate or explode but require an initiator (e.g., heat).
- 2 = Materials that undergo violent chemical reactions at elevated temperatures or pressures or react with water.
- 1 = Materials that are, by themselves, stable but may become unstable at elevated temperatures and pressures.
- 0 = Materials that are stable even under fire conditions and do not react with water.

Health:
- 4 = Short exposure times to these materials are lethal or cause major residual injury.
- 3 = Short exposure times to these materials cause temporary and/or residual injuries.
- 2 = Lengthy (but not chronic) exposure to these materials may cause temporary incapacitation and/or minor residual injury.
- 1 = Materials that, on exposure, cause irritation but only minor residual injury.
- 0 = Materials that, on exposure under fire conditions, offer no more hazard than do ordinary combustible materials.

7.2 GENERAL ANALYTES

7.2.1 General Sample Solvent Considerations

A number of papers have been published dealing with the effect of the injection solvent on peak shape (see 788–790 and previous chapters). Hoffman et al. (788) used benzyl alcohol, tryptophan, cimetidine, and phenylalanine as test solutes. A C_{18} column was used in the studies. Benzyl alcohol was chromatographed using an 20/80 methanol/water mobile phase and 0/100, 50/50, 67/33, and 100/0 acetonitrile/water mixtures as the solute solvent. Peak shape deteriorated as the acetonitrile level increased. At 100% acetonitrile, a distinct double peak was formed. Phenylalanine and tryptophan exhibited similar behavior when the same solute solvents were used but with an 8/92 acetonitrile/water (5 mM phosphate buffer pH = 3.5) mobile phase. Finally, cimetidine exhibited no peak doubling behavior under these conditions. Sample injection size was also studied. The authors provide a detailed explanation (in essence, a complex equilibrium-reaching process) of all the observed phenomena. Vukmanic and Chiba (789) generated similar results for acetonitrile/water solute solvents using a C_{18} column with methyl-2-benzimidazole carbamate and 3-butyl-2,4-dioxo[1,2-*a*]-*s*-triazinobenzimidazole (STB) as test solutes. Their findings emphasized the effect of injection volume. For example, a 10-μL injection of STB yielded one well-shaped peak with acetonitrile/water composition ($\leq 50/50$) as the injection solvent. However, the peak was considerably broader at 50 μL injected and doubled at 100 μL.

Perlman and Kirschbaum (790) prepared a series of solutes (e.g., captopril, nadolol, *o*-nitroaniline, triamcinolone acetate, methylparaben) in neat solvents: acetonitrile, methanol, DMSO, and dichloromethane. These solutions were then injected onto a C_{18} or phenyl column (λ = 214 or 270 nm) and eluted with 50/50 methanol/water or 38.8/1.1/960 methanol/water/dichloromethane mobile phases. Significant differences in the peak areas resulted for some but not all analytes. Deterioration of peak shapes was also common. Prediction of these changes was nearly impossible. For example, *o*-nitoaniline (in methanol) exhibited an increased peak area in a methanol/water, whereas *p*-nitroaniline was unaffected. An awareness of the unexpected and unpredictable effects the sample solvent has on the quantitative results as well and the separation is critical when developing a method.

7.2.2 Simple Substituted Hydrocarbon and Benzene Analytes

Hanai and Hubert (791) conducted an extensive study of the retention characteristics of phenol and 60 substituted phenols (substituent groups included methyl, dimethyl, trimethyl, ethyl, chloro, dichloro, trichloro, tetrachloro, bromo, nitro, dinitro, hydroxy). The log k' values on a C_{18} column were tabulated for mobile-phase compositions ranging from 90/10 to 10/90 acetonitrile/water at 30°C. This is an excellent resource paper for those needing extensive information on the retention of phenols.

An extensive study of the retention of phenols, benzaldehydes, acetophenones, cinnamic acids, and benzoic acids (31 total analytes) was done using a C_{18} column

(λ = 280 nm) and various acetonitrile/water (10 mM phosphate buffer pH = 2.0) gradients (792). This work is also an excellent reference for use in generating separations of similar compounds.

Hanai et al. (793) generated k' values for 43 alkanes, alkylbenzenes, and chloro and polychlorinated benzenes and 41 alkylphenols, chloro and polychlorinated phenols, and bromophenols on a C_{18} column using 60/40–90/10 acetonitrile/water mobile phases. Once again, such a comprehensive tabulation of k' values is extremely valuable for method development work.

In an excellent and thorough study, 15 chlorinated phenols were well resolved on a C_{18} column (λ = 260 nm) using a 31/79 acetonitrile/water (50 mM citrate buffer pH = 4) mobile phase (794). Elution was complete in 90 min. The late-eluting peaks were sufficiently well resolved that a gradient would have been very effective at reducing analysis times. An informative plot of minimum resolution between all analytes versus pH (3–7) versus percent acetonitrile (30–64) was presented.

The retention behavior of 42 substituted benzenes and toluenes (e.g., cyano, nitro, alkyl, hydroxy) was studied on a C_{18} column (λ = 254 nm) using 20/80 and 45/55 acetonitrile/water mobile phases (99). The data are presented as part of log k' graphs.

Fourteen chloro-, chloromethyl-, and dichloroanilines were extracted from water and analyzed on a C_{18} column (λ = 240 nm) using a 36/64 acetonitrile/water (10 mM phosphate buffer pH = 7) mobile phase (795). The samples were preserved with hydrazine sulfate. Without a preservative, up to 70% of the anilines were degraded in 24 h. All components were eluted in 22 min, and overall resolution was quite good, especially considering that a number of unknown peaks eluted as well. Detection limits "well below 0.1 μg/L" were reported.

In an extensive study of amide metabolites of *N*-benzylanilines, Ulgen et al. (796) tabulated the retention times for 43 compounds on a C_{18} column (λ = 254 nm). A series of isocratic acetonitrile/water mobile phases were used. Typically, sets of four or five compounds were studied. For example, benzaldehyde, 2,4,6-trimethylaniline, *N*-benzoyl-2,4,6-trimethylaniline, *N*-(2,4,6-trimethylphenyl)-α-phenylnitrone and *N*-benzyl-2,4,6-trimethylaniline were baseline resolved in 30 min using a 40/60 acetonitrile/water (phosphate buffer pH = 7) mobile phase. The results for six other sets of analytes were tabulated as well. Typical acetonitrile/water (phosphate buffer pH = 7) mobile phase compositions ran from 40/60 to 50/50. Elution times varied between 2.5 and 36 min. If the other separations are anything like the one seen in the only chromatogram shown, this is a very valuable and thorough set of results.

In an interesting study, McCalley (109) studied the effect of the organic mobile-phase constituent (acetonitrile, methanol, or THF) on the peak shapes for 16 basic amine compounds such as pyridine and mono- and di-alkylsubstituted pyridines on a C_{18} column. The mobile-phase compositions were adjusted such that the k' values were at least comparable. It was uniformly shown that a 40/60 acetonitrile/water (37.5 mM phosphate buffer pH = 7.0) mobile phase resulted in slightly lower k' values but consistently higher asymmetry values (i.e., peaks exhibited greater tailing) than did either a 55/45 methanol/water (50 mM phosphate buffer pH = 7) or a 25/75 THF/water (30 mM phosphate buffer pH = 7) mobile phase. The author did not point

to the solvent directly as the cause for such differences; rather he postulated that the solvent effect on the protonation of the solute was the driving factor. Hence, if the solute were more likely protonated in the acetonitrile mobile phase, then the solute would undergo strong hydrogen-bond interactions with the surface silanol groups. The solvent system giving the best results (in terms of peak symmetry) was the one containing THF. This should not be surprising since it is well known that THF sharpens peaks when added at low volumes (2–10%) to mobile phases.

An extensive table of log k' values for 32 phenols and substituted phenols (nitro, chloro, bromo, alkyl), benzene and substituted benzenes (alkyl, chloro, bromo), and naphthalene, anthracene, pyrene, and chrysene was generated by Bosch et al. (86). Mobile-phase compositions covered the entire 100% water–100% acetonitrile range. No values were generated for log k' values > 2 for a C_{18} column. Although these data were used to help generate a predictive retention scheme, the results are an excellent resource for use in method development.

Ryba (797) used *N*,*N*-dimethylformamide (DMF) as a mobile phase component in analyses in which *N*-heterocyclic compounds such as pyridine, pyrimidine, and purine derivatives were separated. In a very instructive application, phenol, pyridine, 3-picoline, 2,4-dimethylphenol, and quinoline were separated on a C_{18} column. One mobile phase used was 60/40 methanol/water. Here, the amine peaks were severely tailed, poorly resolved, and retained for up to 11 min. The same analytes with a 50/10/40 methanol/DMF/water mobile phase exhibited only slight peak tailing and complete resolution and elution in less than 8 min. In a similar fashion, 30 test solutes were chromatographed and their k' values and peak asymmetries tabulated. Without exception, the amine compounds exhibited a significant improvement in peak shape in the DMF-containing mobile phase. Other polar compounds (e.g., benzophenone, *o*-cresol, nitrobenzene) were not affected in this manner.

7.2.3 Other Organic Compounds

The 2,4-dinitrophenylhydrazine derivatives of acetone, methyl ethyl ketone (MEK), cyclohexanone, *t*-butyl methyl ketone, adamantanone, and 4-methylbenzophenone were baseline resolved on a C_{18} column (laser source detector at 442 nm) using a 75/25 acetonitrile/water mobile phase (798). Elution was complete in 60 min. With the specialized detector, 120-fmol detection limits (S/N = 3) were reported.

Acrolein, crotonaldehyde, and methacrolein were separated as their anthrone derivatives on a C_{18} column (λ = 405 nm, ex; 480 nm, em) using a 60/40 acetonitrile/water mobile phase (799). Elution was complete in 10 min and peak shapes were very good. Detection limits of 5 ppb and linear concentration ranges from 20 ppb–14 ppm were reported.

A series of alkanals and alkenals, degradation products of polyunsaturated fatty acids, were separated as their dabsylhydrazine derivatives on a C_{18} column (λ = 436 nm) using a 60-min acetonitrile/water gradient (40/60–80/20) (800). Acrolein, propionaldehyde, crotonaldehyde, butyraldehyde, 4-hydroxy-2-nonenal, malonaldehyde, *trans*-2-pentenal, hexanal, heptanal, *trans*-2-octenal, octanal, *trans*-2-nonenal, and nonanal were baseline resolved. When malonaldehyde and

trans-2-hexenal were included in the set, they coeluted with pentanal. Peak shapes were excellent, and detection limits (S/N = 4) of 5 ng injected were reported.

The reaction products of α-acetoxy-*N*-nitrosopyrrolidone and α-acetoxy-*N*-nitrosopiperidine with DNA were characterized on a C_{18} column (λ = 254 nm) using a 30 min acetonitrile/water (10 mM phosphate buffer pH = 7.0) gradient (0/100–30/70) (801). The products, N^2-(tetrahydofuran-2-yl)deoxyguanosine and N^2-(3,4,5,6-tetrahydro-2*H*-pyran-2-yl)deoxyguanosine, were well resolved from DNA fragments. Elution was complete in < 35 min.

The retention characteristics of 4,4′-disubstituted benzanilides and benzamides (chloro, methyl, methoxy, nitro) were conducted on a C_{18} column (λ = 254 nm) using a series of acetonitrile/water mobile phases (802). The contribution of the substituent to retention was tabulated, and a predictive model for the calculation of log *k′* values was presented. Mobile-phase compositions from 35/65 to 70/30 acetonitrile/water were used, and a plot of log *k′* values (calculated) versus log *k′* (actual) was also presented.

7.2.4 Organometallics and Metal–Ligand Complexes

Selenols (RSeH), diselenides (RSe–SeR), and selenyl sulfides (R′S–SeR) were separated on a C_{18} column (electrochemical detector at -1.10 V vs. Ag/AgCl). A 5/95 acetonitrile/water (50 mM phosphate buffer pH = 2.9 + 40 mg/L sodium octyl sulfate) mobile phase was used (803). A plot of the *k′* value versus percent acetonitrile for five such compounds was presented for the acetonitrile range of 1–8%. The *k′* values ranged from 4 to 50 at 1% acetonitrile to 0.1–4 at 8% acetonitrile. Selective detection can be achieved by setting the potential to –0.55 V (for diselenides and selenyl sulfides) or +0.15 V (for selenols only).

Trace levels of vanadium (V) were extracted from water and coal fly ash samples and analyzed as the 2-(8-quinolylazo)-5-(dimethylamino)phenol complex (804). A C_{18} column (λ = 550 nm) and a 50/50 acetonitrile/water (10 mmol/kg tetrabutylammonium (TBA) bromide) mobile phase generated the separation. A number of potentially coeluting metal ions were studied as well: Ni, Co, and Fe. Detection limits of 3 pg were reported. Vanadium (IV) and (V), Pb(II), Cu(II), and Cr(VII) were resolved on a C_8 column (λ = 245 nm) with a 12/78 acetonitrile/water (50 mM TBA + 2 mM EDTA to pH = 6) mobile phase (805). Elution was complete in < 20 min, and peak shapes were good. Detection limits of 1 ng and linear concentration ranges of 1–30 μg/mL were reported.

Gold (I) was quantitated as its cyano complex by HPLC using a C_{18} column (λ = 214 nm) and a 32/68 acetonitrile/water (5 mM TBA) (806). The complex eluted in 8 min. Pd(II) and Pt(II) were resolved from Au(I) when the acetonitrile level was decreased to 23%. In this case elution was complete in 35 min. The detection limit for Au(I) was reported as 0.4 ppb.

Four organomercury compounds (methoxyethyl, ethyl, phenyl, and methyl) were extracted from water and separated from inorganic mercury (Hg^{2+}) as their pyrrolidinethiocarbamate (PDTC) complexes (807). A C_{18} column (λ not given) and a 60/40 acetonitrile/water (5 mM sodium PDTC to pH = 5.5 with ammonium acetate)

resolved these compounds in < 10 mins. Detection limits (direct injection) of 5 ng were reported.

7.2.5 Summary

Acetonitrile is successfully used for the separation of a wide range of analytes, both polar and nonpolar. Typically its inclusion in the mobile phase results in sharper peaks and shorter retention times as compared with alcohols. Acetonitrile is the most commonly used RP solvent because of these facts and the unique set of properties it brings to HPLC: low UV cutoff and low viscosity mixtures when used with water.

7.3 ENVIRONMENTALLY IMPORTANT ANALYTES

7.3.1 Substituted Benzenes and Related Analytes

Alarcón and co-workers (808) successfully separated nine priority pollutant substituted phenols (e.g., 2,4-dimethyl, 2-chloro, 2- and 4-nitro, 2,4,6-trichloro) from phenol using a C_{18} column ($\lambda = 280$ nm) and a 34/10/56 acetonitrile/methanol/water [30 mM ammonium acetate buffer pH = 5.0 + 0.15 mM cetyltrimethylammonium bromide (CTAB)] mobile phase. Good resolution was obtained, but this result was noted to be very dependent on the C_{18} column/CTAB concentration combination used. Elution was complete in < 15 min.

Guaiacol, bromoxynil, ioxynil, trichlorosyringol, *o*-chlorovanillin, dinoseb, and 11 chloro-, nitro-, and methyl-substituted phenols were baseline resolved on a C_{18} column using a 24-min (90/10 acetonitrile/methanol + 0.015% TFA)/water (0.05% TFA) gradient (33/64–75/25). The monitored wavelength was changed from 280 to 230 nm at the 7.8-min mark. Peak shapes were uniformly excellent. Detection limits were < 20 ng injected for each analyte. With a pre-extraction process described in the paper, overall detection limits were reported as 0.1 μg/L (809).

Six airborne organic pollutants—2,4- and 2,6-toluenediamine, 2-amino-4-isocyano and 2-amino-6-isocyanotoluene, and 2,4- and 2-6-toluenediisocyanate—were collected and baseline resolved on a C_{18} column ($\lambda = 254$ nm or electrochemical detection +950 m V vs. Ag/AgCl) using a 37/67 acetonitrile/water (0.1 M phosphate buffer pH = 7.0) mobile phase (810). The isocyanate substituents were derivatized with ethylchloroformate to generate toluene–urethane compounds. Elution was complete in 15 min. A set of plots of k' versus percent acetonitrile was presented for all analytes. Detection limits of 0.05 pg/μL were reported. The working range was reported as 2–1000 μg/m^3 of air.

Cigarette smoke was analyzed for aldehydes and ketones via derivatization with dinitrophenylhydrazine. The separation of formaldehyde, acetaldehyde, acetone, acrolein, and propionaldehyde was achieved on a C_{18} column ($\lambda = 345$ nm) using a 60/40 acetonitrile/water mobile phase (811). Elution was complete in 12 min. A similar method was used (96) on a slightly expanded set of analytes (including butyraldehyde and benzaldehyde) once again on a C_{18} column ($\lambda = 365$ nm) using a

65/35 acetonitrile/water mobile phase. Elution in this study was complete in < 20 min. Detection limits were 1.5 pmol, and the standards covered the 30-μg/mL–30-mg/mL (10 μL injected) range.

Thirteen chloroanilines (two chloroaniline isomers, five chloromethylaniline isomers, five dichloroaniline isomers, and 5-chloro-2-hydroxyaniline) and aniline were extracted from river and drinking water samples (812). They were baseline resolved on a C_{18} column (λ = 245 nm or electrochemical detection at +0.95 V vs. Ag/AgCl) using a complex 60-min acetonitrile/water (50 mM acetate buffer pH = 4.9) gradient (26/74–40/60). Excellent resolution and peak shapes were obtained. Detection limits (S/N=5) were reported as 15–25 ng/L with UV detection and 3–5 ng/L for electrochemical detection. A table of analyte λ_{max} and pK_a values was presented.

7.3.2 PAHs, Substituted PAHs, and Related Analytes

The standard PAH separation is EPA Method 610 (or SW-846 8310). It calls for the use of a C_{18} column (λ = 254 or 280 nm, ex; 389-nm UV cutoff, em) and a 25-min acetonitrile/water (~40/60–100/0) gradient to separate 16 priority pollutants (naphthalene to indeno[*1,2,3-cd*]pyrene). Fluorescence detection limits of < 0.1 μg/L are cited.

Wise et al. (813) recently reviewed polyaromatic hydrocarbon (PAH) analyses. Both monomeric and polymeric C_{18} columns were studied with respect to retention and separation characteristics. A typical method was a 30-min 40/60–100/0 acetonitrile/water gradient with λ = 254 nm. A large number of references that include slight variations of this and the EPA 610 scheme are referenced within this review.

A series of 12 isomers of PAHs with molecular-weight 302 were well resolved in 30 min on a polymeric C_{18} column (λ = 254 nm) using a 10-min acetonitrile/water gradient (90/10–100/0 and hold) (814). Similarly, 11 isomers of PAHs with molecular weight 278 (e.g., benzo(*c*)chrysene, pentacene) were separated on the same column using a 15-min acetonitrile/water gradient (81/15–100/0). Peaks were slightly tailed, most likely due to mass transfer effects of the large nonpolar molecules into and out from the polymeric support. A monomeric C_{18} support, which would not be expected to exhibit these effects as strongly, did not resolve either of these sets of compounds.

A series of methylated chrysenes, picenes, and perylenes were separated on a C_{18} column (λ = 265 nm, ex; 365 nm, em) at 28 °C using an 80/20 acetonitrile/water (for chrysenes) or a 95/5 acetonitrile/water (for picenes and perylenes) mobile phase (815). The authors noted that, contrary expectations, some of the methylated solutes eluted *prior* to their unsubstituted analogs. A chromatogram supporting this showed that 5-methyl- and 6-methylchrysene eluted before chrysene on a Bakerbond C_{18} column. This behavior was attributed to a combination of the length:width ratio of the solute, the position of the methyl substituent (both on the rings and with respect to the ring plane), and the characteristics (pore size, carbon loading) of the support material.

The retention of 34 nitrated PAH compounds (e.g., quinolines, naphthalenes, biphenyls, anthracenes, fluorenes) was studied on a C_{18} column using mobile phases

consisting of 50/50, 60/40, and 70/30 mixtures of acetonitrile/water at 35, 45, 55, and 65°C (171). Samples containing 1 mg/mL of each compound, and 1-μL aliquot, were injected. The log k' value was tabulated for each compound under each mobile phase/temperature combination. This is an excellent reference for those seeking detailed information on the retention behavior of nitroPAHs.

The deoxyadenosine and deoxyguanosine adducts of *syn*-benzo[*g*]chrysene-11,12-dihydrodiol-13,14-epoxide were baseline resolved on a C_{18} column (λ = 264 nm) with a 50-min acetonitrile/water/methanol gradient (25/75/0–12/60/28)(839). The *R* and *S* conformational adducts were separated individually using a 24/76 acetonitrile/water mobile phase. Elution required nearly 120 min in all cases, and peaks were quite tailed.

Six hepatic metabolites of 7,9- and 7,10-dimethylbenz[*c*] acridine (e.g., 7-(hydroxymethyl)-10-methylbenz[*c*]acridine, 7,9-dimethylbenz[*c*]acridine-5,6-oxide) were separated from the parent compounds on a C_{18} column (λ = 270 nm) using a complex 80-min acetonitrile/water gradient (24/76–100/0) (502). Analytes eluted from 35 to 75 min. Peak shapes were excellent, even at the long retention times. Peaks of interest were well separated from other extracted compounds.

7.3.3 Nitro-, Nitroso-, and Chlorinated Nonpesticide/Herbicide Analytes

The anaerobic metabolites of 2,4-dinitrotoluene and 2,6-dinitrotoluene (2,6-diaminotoluene, 2-hydroxyamino-6-nitrotoluene, 2-nitroso-6-nitrotoluene, 2-amino-6-nitrotoluene, 3,3′-diamino-2,2′-dimethylazoxybenzene, 2,2′-dimethyl-3,3′dinitroazoxybenzene) were baseline resolved on a C_{18} column (λ = 250 nm) using a complex 65-min acetonitrile/water gradient (15/85–60/40) (817). Detection limits of 5 ng injected were reported. A linear concentration range of 5–50 ng injected was generated.

The biological degradation of 2,4,6-trinitrotoluene (TNT) was followed by Ahmad and Roberts (818) using a C_{18} column (photodiode array detector, λ = 200–600 nm) and a complex 18-min acetonitrile/water (50 μL/L H_3PO_4 to pH = 3.2) gradient (10/90–100/0). Phloroglucinol (1,3,5-benzenetriol), pyrogallol (1,2,3-benzenetriol), methyl phloroglucinol, *p*-cresol, 2,2′,6,6′-tetranitro-4,4′-azoxytoluene, 4, 4′6, 6′-tetranitro-2, 2′-azoxytoluene, and the typical reduced-form nitrotoluenes (e.g., 2-amino-4,6-dinitrotoluene) were separated from TNT and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) in $<$ 20 mins. Detection limits of 10 ng injected (for the benzenetriols) and down to $<$ 1 ng injected for the nitro-substituted compounds were reported. Although the overall chromatography was reported to be independent of pH (at least around pH 3.2), the stability of 2,4,6-triaminotoluene (TAT) was such that at pH = 6.0 it eluted as five peaks, four peaks at pH = 4.0 and two peaks (eluting near the void volume of the system) at pH = 3.2. The stability of the TAT was then examined in the standard solutions, and it was found that the TAT could be eluted as one peak when the standard solution was made up in 50/50 acetonitrile/methanol.

Four bacterial metabolites of 2,6-dinitrotoluene (2-amino-6-nitrotoluene, 2-nitroso-6-nitrotoluene, 2-hydroxyamino-6-nitrotoluene, 2,2′-dimethyl-3,3′-dinitroazoxybenzene) were baseline resolved on a C_{18} column (λ = 250 nm)

using a complex 70-min acetonitrile/water gradient (15/85–65/35) (819). Linear response ranges of 10–150 ng injected were obtained. Peak shapes were very good, and elution was complete in 60 min.

Some of the most volatile priority pollutants that have been quantitated by HPLC are the *N*-nitrosoamines. The dimethyl-to dibutyl-, piperidyl-, and pyrrolidyl-substituted *N*-nitroso compounds were well resolved as their 4-(2-phthalimidyl)-benzoyl chloride derivatives (820). A C_{18} column (λ = 299 nm, ex; 426 nm, em) and a 48/52 acetonitrile/water mobile phase were used for the separation. Elution was complete in $<$ 20 min Detection limits (S/N = 3) of ~1 pmol injected for each analyte and working ranges of 0.16–100 nmol/L were reported. A plot of k' versus percent acetonitrile (40–70%) was shown for these analytes. The k' values for the dipropyl and dibutyl analytes were well over 5 when the acetonitrile level dropped below 50%.

The methyl, hydroxy, methoxy, chloro, cyano, and nitro derivatives of *N*-nitroso-*N*-methylaniline were separated on a C_{18} column (λ = 290 nm) using a 30/70 acetonitrile/water (10 mM phosphate buffer pH = 5.6) mobile phase (821). The methyl- and chloro-derivative peaks were noticably fronted. Elution was complete in 30 min. The same separation was achieved in $<$ 15 min with excellent peak shapes, when the mobile phase was changed to 200/200/200/3 acetonitrile/methanol/water/80% H_3PO_4 (at pH = 3.2). A low enough mobile phase pH seems to be necessary to prevent unfavorable analyte interaction with the silica support. Plots of log k' versus percent acetonitrile (20–50%) were nonlinear. The detection limits were reported as 1 ng/3 μL (3×10^{-6}M) sample injected. Linear ranges were found to cover the range of 5×10^{-6}M to 2×10^{-4}M.

The *syn* and *anti* conformers of *N*-nitrosopipecolinic acid, *N*-nitrosonipecotic acid, *N*-nitrosothiazolidine-4-carboxylic acid, and *N*-nitrososarcosine were individually baseline resolved on an α-cyclodextrin column (λ = 238 nm) using a 90/10 acetonitrile/water (10 mM triethylammonium acetate) mobile phase (706). A 10-μL aliquot of 0.5-μg/μL standards was injected. No resolution of conformers was achieved at room temperature, but when the system temperature was decreased to $-8°$, good resolution was obtained. Peak shapes were good, and elution times ranged from 6 to 20 min (analyte dependent).

7.3.4 Pesticide and Herbicide Residue Analytes

A chromatogram showing the baseline separation of 21 National Survey Pesticides was generated by Miles (209) using a C_{18} column (conductivity detector) and a 60-min acetonitrile/water gradient (30/70–60/40). Peaks of 50 ng were easily detected.

Although Parilla et al. (822) reported the quantitation of fenamiphos and folpet on a C_{18} column (photodiode array detector, λ = 210, 224, or 252 nm), the method developed actually baseline resolves 22 pesticides (e.g., metomyl, aldicarb, dichlorvos, diuron, methiophos, iprodione, chlorfenvinphos, α- and β-endosulfan, carbophenothion) in 25 min. A 20 min acetonitrile/methanol/water (27/17/56 $\rightarrow$ 90/5/5) gradient was used. Peak shapes were excellent, and representative chromatograms plus tabulated results of retention times for each pesticide were presented. In a similar

study, Parilla et al. (823) developed a screening method for 21 pesticides (same set as above) in water using the C_{18} column (λ = 212, 220, 250, and 330 nm) and the same gradient program. Linear working ranges and detection limits are tabulated for all the pesticides used in the study.

An excellent separation of 28 pesticides was generated by Huen et al. (824). All compounds were baseline resolved on a C_{18} column (λ = 234 nm or photodiode array detector) using a 40-min acetonitrile/water (+1 mL H_3PO_4/L) gradient (10/90–90/10). The test compounds included metamitron, diuron, atrazine, metazachlor, neburon, pyrazaphos, chlorpyriphos, trifluralin, and fenpropathrin. Detection limits for drinking-water samples were reported to be 0.01 μg/L.

In an excellent study, 33 pesticides (including triazines, phenylureas, dinitroanilines, acetanilides, thiocarbamates, and phosphorthioates) were extracted from water and resolved on a C_{18} column (λ = 220 nm). A nonlinear 85-min acetonitrile/water (1 mM ammonium acetate) gradient (20/80–85/15) was used (825). Concentrations of the analytes varied from 250 mg/mL to 1.1 μg/mL (20 μL injected). Detection limits (S/N = 6) ranged from 6 ng/mL (napropamide) to 163 ng/mL (phenylurea). In the same study, a set of nine acidic phenolic pesticides (thifensulfuron-methyl, metsulfuron-methyl, dicamba, MCPA ≡ [4-chloro-2-methylphenoxylacetic acid], MCPB ≡ [4-chloro-2-methylphenoxylbutyric acid], bromoxynil, dichlorprop, ioxynil, bifenox acid, haloxyfop) were also studied. Here, a C_{18} column (λ = 229 nm) was also used and a 16-min acetonitrile/water (17 mM H_3PO_4) gradient (48/52–60/40) generated a baseline resolution of the analytes. Detection limits od 25–80 ng/L were reported.

Fifty-two nonvolatile pesticides (including carbamates, triazines, phosphoramides, chloro-*N*-substituted amides, phthalate esters) were extracted from aqueous samples and analyzed using a C_{18} column (volatile salt ionization thermospray MS) and a 33-min acetonitrile/water (0.1 M ammonium acetate) gradient (20/80–50/50) (826). Detection limits were reported to fall in the 1–20 μg/L range (analyte-dependent). These data were presented in a tabular format. In addition, a separate 25-min acetonitrile/water (0.01 M ammonium acetate + 0.1 M ammonium formate) gradient (20/80–60/40) was run and retention times for all 52 compounds were tabulated. Retention times ranged from 4 to 34 min.

Seventeen organophosphate pesticides (e.g., famphur and famphur oxon, paraoxon, fenthoxon, ronnel, 3-chloro-4-methyl-7-hydroxycoumarin, stirofos, parathion, coumphos) were extracted from beef tissues and baseline resolved on a C_{18} column (photodiode array detector, λ = 190–350 nm). A complex 34 min acetonitrile/water (55/45–70/30) gradient was used (827). Excellent peak shapes were obtained. A table of ϵ_{max}, recoveries, and detection limits (0.25–1.0 ppm, analyte-dependent) was presented for all analytes.

Schlett (828) analyzed drinking and surface water samples for pesticide residues after extraction and concentration. Good resolution for some 20 pesticides was obtained on a C_{18} column (photodiode array detector, λ = 200–300 nm) using an 82-min acetonitrile/water (2 mM sodium acetate) gradient. A table of max for 69 compounds was included in the study. Detection limits of < 0.025 μg/L and linear concentration ranges of 0.025–1 μg/L were reported.

Pentachlorophenol and three of its biodegradation products (pentachloroanisole, tetrachloro-*p*-hydroquinone, tetrachloro-*p*-benzoquinone) were baseline resolved in 18 min on a C_{18} column (λ = 254 nm) using an isocratic 60/40 acetonitrile/water (0.1% acetic acid) mobile phase (178). This was considerably more efficient than a methanol/water gradient, not because of better resolution or shorter elution times, but because there was no re-equilibration time needed between injections for the acetonitrile/water system.

The phenyl sulfone, sulfoxide, and sulfonic acid metabolites of fenamiphos were extracted from soil and analyzed using a C_{18} column (λ = 240 nm) and a 50-min acetonitrile/water (1 g/L acetic acid) gradient (0/100–50/50 at 30 min to 100/0 at 50 min) (829). Detection limits of 10 ppb and a linear concentration curve up to 500 ppb were reported.

Dinitroaniline herbicides (dinitramine, ethalfluralin, trifluralin, pendimethalin, and isopropalin) were extracted from soil and water matrices and baseline resolved on several RP columns, including C_{18}, C_8, C_6, and C_1 (λ = 220 nm) (830). Good resolution was obtained using a C_{18} column and a 55/45 acetonitrile/water mobile phase. Similar success was achieved with a 25/75 acetonitrile/water mobile phase and a C_1 column. Both methods gave complete elution in $<$ 20 min Detection limits were reported as 20 μg/kg in soil samples and 0.5 μg/L in water samples.

Twenty-six methyl carbamate residues and metabolites (e.g., aldicarb sulfoxide and sulfone, aldicarb, carbaryl, methiocarb and its sulfoxide and sulfone, etrofolan, baycarb, butocarboxim, and thiofanox) were extracted from plant and soil matrices and analyzed as their *o*-phthalaldehyde derivatives using a C_{18} column (λ = 340 nm, ex; 455 nm, em) (831). A 15-min acetonitrile/water (20/80–80/20) gradient was used. Carbofuran, propoxur, and bendiocarb coeluted. Peaks corresponding to 10-ng injected were readily detected. Propoxur, carbofuran, and bendiocarb were resolved using a separate shallow 15 min acetonitrile/water gradient (35/65–40/60).

Aldicarb and four soil degradation compounds (the oxime, sulfoxide, sulfone, and sulfoxide oxime) were baseline resolved on a cyanopropyl column (λ = 210 nm) using a 20/80 acetonitrile/water (acetate buffer pH = 6.0) mobile phase (194). Elution was complete in 8 min. Retention times were tabulated for 15, 20, and 25% acetonitrile in water mobile phases. The detection limits were reported for each compound at wavelengths of 210–254 nm. At 220 nm, detection limits were reported as 50–200 μg/L (analyte-dependent).

Ethyl *N*-phenylcarbamate, 4,4′-methylenebis(ethyl phenylcarbamate), 4,4′-methylenebis(phenyl isocyanate), and 10 decomposition products were separated as their urea derivatives on a C_{18} column (λ = 254 nm) using a 75/25 acetonitrile/water (1% TEA to pH = 3.0 with H_3PO_4) mobile phase (832). Very good resolution and peak shapes were obtained. Elution was complete in 30 min, and detection limits were reported to be 1–7 ng injected (analyte-dependent).

Newsome et al. (833) studied the chromatography of 11 carbamate pesticides (e.g., aldicarb, aldicarb sulfoxide and sulfone, oxamyl, carbofuran, and 3-hydroxy- and 3-ketocarbofuran) on a C_8 column. A postcolumn derivatization detection technique (with *o*-phthalaldehyde) (λ = 336 nm, ex; 440 nm, em) was compared with an atmospheric-pressure ionization MS detector. A 20-min acetonitrile/water gradient

(12/78–70/30) generated good resolution and peak shapes. Detection limits for all compounds were tabulated and were ~ 0.1 ng for fluorescence and 1 ng for API-MS.

Kettrup and co-workers (834,835) published two studies on the separation of triazine herbicides using C_{18} columns and acetonitrile/water mobile phases. In the first study, 11 triazines (e.g., atrazine, simazine, prometryne, ametryne, atraton, and prometon) and seven of their metabolites (e.g., hydroxysimazine, hydroxyatrazine, and hydroxyterbuthylazine) were separated using a 15-min acetonitrile/water (1 mM phosphate buffer pH = 7.0) gradient (18/82–63/37). Very good resolution of the peaks resulted. A plot of k' versus percent acetonitrile for 12 selected components was shown. A water sample was spiked to the 0.0–2 mg/L level, extracted, concentrated, and subsequent analysis showed each analyte was easily detectable (λ = 220 nm). The second study used a subset of these compounds (desisopropylatrazine, desethylatrazine, simazine, atrazine, and erbuthylazine), which were analyzed via a column switching technique. The final separation was achieved on a C_{18} column (λ = 220 nm) using an 18-min acetonitrile/water (1 mM phosphate buffer pH = 7.0) gradient (20/80–45/55). With a 100-mL enrichment sample, detection limits of 15 ng/L were reported.

Simazine, atrazine, propazine, and terbuthylazine were extracted from water, baseline resolved, and separated from 3-chloro-4-methoxyaniline, 3-chloro-4-methylaniline, 4-isopropylaniline, and 3,4-dichloroaniline on a C_{18} column (λ = 230 nm) using a complex 60-min acetonitrile/water (50 mM $HClO_4/LiClO_4$ buffer pH = 4) gradient (18/85–46/54) (829). Peak shapes were excellent, and detection limits of < 10 ng/L were reported.

Propazine, atrazine, simazine, and six metabolites were baseline resolved on a C_{30} column (photodiode array detector, λ = 200–356 nm) using a 32-min acetonitrile/water (1 mM phosphate buffer pH = 7.0) gradient (15/85–70/30). Concentrates of water extracts gave < 10-ng/L detection limits (837).

Steinheimer (838) studied atrazine and related compounds, deisopropylatrazine, deethylatrazine, and terbuthylazine in soil and water samples. These compounds were resolved using the following conditions: C_{18} column (λ = 220 nm, or photodiode array detector, λ = 220–254 nm) and a complex 23-min acetonitrile/methanol/water (10/0/90–100/0/0–0/100/0) gradient. The last peak of interest eluted at 17 min. Acetonitrile and methanol flushes were done to clean the system between injections. Calibration curves are shown for four of the analytes and were linear over the 0.2–50-ppm range. Detection limits of extracted samples were reported as 0.4 μg/L (water) and 40 μg/kg (soil). Solubility in water was given for the components; the lowest was terbuthylazine at 8 ppm.

Atrazine and four metabolites generated from topical applications (2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine, 2-chloro-4-ethylamino-6-amino-*s*-triazine, 2-chloro-4-amino-6-isopropylamino-*s*-triazine, 2-hydroxy-4,6-diamino-*s*-triazine) were resolved on a C_{18} column (λ = 223 nm) (839). Elution was complete in 10 min when a 14-min acetonitrile/water gradient (20/80–50/50) was used. The assay was linear at 2–120 μg/mL (20-μL injections). Peak shapes and resolution were good.

Barceló (517) analyzed 2,4-D, 2,4,5-T, Silvex, ethylatrazine, isopropylatrazine, and atrazine on a C_{18} column (positive ion thermospray MS) using a 50/50

acetonitrile/water (50 mM ammonium acetate) mobile phase. The chloroatrazines produced primary ions that were acetonitrile adducts. Adducts were not seen when cyclohexane was used as the mobile phase (see Chapter 3).

The herbicide hexazinone and six soil and vegetation metabolites (desisopropylatrazine, hydroxyatrazine, desethylatrazine) were baseline resolved in 30 min using a C_{18} column (photodiode array detector, λ = 200–254 nm and thermospray MS) at 40°C (840). A complex 50-min acetonitrile/water (50 mM ammonium acetate) gradient (8.5/91.5–70/30) produced excellent peak shapes. Detection limits of 50–400 ppb were reported (analyte-dependent).

Benzothiazole, 2,2′-(dithiobis)benzothiazole, and 2-mercapto-, 2-(methylthio)-, and 2-(thiocyanomethylthio)benzothiazole were baseline resolved on a C_{18} column (λ = 250, 380, or 325 nm) using a complex 20-min acetonitrile/water (4 mM NaH_2PO_4 buffer pH = 4.5) gradient (63/37–90/10). Peak shapes were excellent. Extraction from industrial wastewater samples gave detection limits in the low-nanogram (0.1–10 ng) injected range (841).

Paraquat and diquat extracted from high moisture crops were analyzed on a silica column (photodiode array detector, with λ = 257 and 310 nm for paraquat and diquat, respectively) (842). A 40/60 acetonitrile/water (5.0 g NaCl to pH = 2.2 with HCl) mobile phase was used. The use of methanol in place of acetonitrile produced very broad and tailed peaks. This separation was compared with a separation system that consisted of an aminopropyl column and a ternary acetonitrile/methanol/water (NaCl/HCl system) mobile phase. The latter system took $>$ 2 h to equilibrate whereas the silica system was ready for use in 15 min. A plot of k' versus percent acetonitrile (10–70%) was U-shaped, and excessive peak tailing occurred at levels of $<$ 30 % and $>$ 50 % acetonitrile. The authors especially noted that the NaCl/HCl concentrations used were minimized because of the corrosive effects of chloride salts and acids on LC hardware. Detection limits of 10 ppb and linear working ranges of 2–500 ng injected were reported.

The isomers of tetrachlorodibenzo-*p*-dioxin were separated on both monomeric and polymeric C_{18} columns (λ = 235 nm) using a 88/12 acetonitrile/water mobile phase (180). Eleven discernible peaks were generated on the polymeric phase (which gave superior separation). Each sample mixture was a 7-μL injection containing ~3 ng of each isomer. Peak shapes were good, and elution was complete in 35 min.

1-Naphthylamine and the pesticides naptalam (*N*-naphthylphthalamic acid) and antu (1-naphthylthiourea) were extracted from river water samples and quantitated on a C_{18} column (λ = 220 nm) using a 45/55 acetonitrile/water (0.1% acetic acid) mobile phase (843). Elution was complete in $<$ 5 min, and good peak shapes were obtained. Retention times and resolution factors for isocratic mobile phases in the range 20–50% acetonitrile were also tabulated. The authors noted that significant noise levels were obtained when the detection wavelength was set at 215 nm, most likely due to the acetic acid. A detection limit of ~30 ppb was reported.

Seven pyrethroid insecticides (allethrin, cypermethrin, tetramethrin, bifenthrin, permethrin, fenpropathrin, and fenvalerate) extracted from surface water samples were baseline resolved on a PAH column (λ = 235 nm) at 40°C using a 20-min acetonitrile/water gradient (35/65 (12.5-min hold)–80/20) gradient (844). The authors

note that the analytes can adsorb to storage vessel walls, and this loss was avoided through the addition of 1×10^{-3}M Brij-35. Too high a level of Brij-35 caused breakthrough problems during the chromatography. Detection limits (S/N = 3) were reported to be between 0.05 and 0.4 μg/L.

Methoxychlor and monohydroxy-, bishydroxy-, ring hydroxylated-, and trishydroxymethoxychlor were extracted from liver tissue and resolved on a C_{18} column (λ = 228 nm) in 16 min using a 50/50 acetonitrile/water mobile phase (845). Good resolution and peak shapes were obtained.

Five superwarfarin rodenticides (chlorophacinone, bromadiolone, difethialone, and *cis*- and *trans*-brodifacoum) were extracted from serum and separated on a C_8 column (λ = 285 or 265 nm, ex; 400 nm, em) (846). Very good peak shapes were generated with a 45/25 acetonitrile/water (20 mM acetate buffer pH = 4.7 with 1mM tetrabutylammonium hydroxide) mobile phase. Detection limits for UV were 20–75 ng/mL; for fluorescence, 3-12 ng/mL (however, chlorophacinone and difethialone did not fluoresce). Concentration curves were linear from 100 to 1000 ng/mL.

7.3.5 Summary

Acetonitrile has been used as the organic mobile-phase constituent for nearly every separation of environmentally important analytes. Often, acetonitrile gives not only the best peak shape but also the shortest retention times and best resolution. The one case where this does not apply is in the separation of hydroxylated compounds (e.g., phenol). With these compounds, the addition of methanol almost always improves the overall efficiency of the separation.

7.4 INDUSTRIAL AND POLYMER ANALYTES

7.4.1 Surfactant and Additive Analytes

Methyl-, ethyl-, propyl-, and butylparabens were extracted from various pharmaceutical formulations and quantitated using a C_{18} column (λ = 230 nm) and a 40/60 acetonitrile/water (6.5 mM NaH_2PO_4 buffer pH = 5.5) mobile phase (847). Elution was complete in < 7 min, and complete resolution was obtained. Injections of 10-μL aliquots of standards ranging from 10 to 30 μg/mL were made. Detection limits (S/N = 3) of ~1 μg/mL (10 μL injected) were reported.

Polymer additives Irgafos P-EPQ and Irgafos 168 were analyzed on a C_{18} column (λ = 220 nm) using a 65/35 acetonitrile/chloroform mobile phase (644). Elution was complete in 10 min. Irganox 1010, Irganox 1076, and Sumilizer BHT were analyzed similarly but with a 65/35 acetonitrile/acetone mobile phase. A set of four antioxidants (Santowhite, Irganox 3114, Irganox 1010, Irganox 1330) were baseline resolved on a C_{18} column (λ = 280 nm) using a 100% acetonitrile mobile phase (848). A 20-μL injection of 5-μg/mL standards gave readily detected peaks. Elution was complete in 12 mins Peak shapes were excellent.

Stilbene, biphenyl stilbene, pyrazoline, oxazole, and coumarin-based brighteners used in detergent formulations were separated on a C_8 column and monitored by UV (λ = 340 nm), fluorescence (λ = 340 nm, ex; 390 nm, ex), and API MS (849). A table of relative ion intensities for the MS detector showed that a 95/5 acetonitrile/water mobile phase gave the highest ion intensity whereas a 50/50 acetonitrile/water mobile phase caused a 16–95% reduction in ion intensity, and a 50/50 acetonitrile/water (5 mM ammonium acetate) mobile phase resulted in a range from a 10-fold increase to an 85% reduction in ion intensity. A set of samples ranging from 3 to 15 ppm (20 μL injected) was used for working-curve generation.

A set of 15 sunscreen agents [(e.g., 2,4-dihydroxybenzophenone, 3-benzylidenecamphor, cinnamic acid, 2-ethylhexyl-4-dimethylaminobenzoate, phenyl salicylate, 5-(3,3-dimethyl-2-norbornylidene-3-penten-2-one)] were extracted from lotions and separated on a C_{18} column (λ = 280 and 313 nm) at 30°C using a 40-min acetonitrile/water (1 mM $HClO_4$/5 mM $NaClO_4$) gradient (15/85–90/10) (850). Peak shapes were excellent, but complete resolution of all compounds was not achieved.

Nonylphenol ethoxylate surfactants were extracted from waste water samples and analyzed on both cyanopropyl and C_{18} columns (λ = 229 nm) (851). For oligomers of 1–3 ethoxy units, a 98/2 acetonitrile/water mobile phase and the C_{18} column were used. Peaks were broad, especially the trimer, most likely because of slow mass transfer effects. Elution was complete in 15 mins. For oligomers of 4–14 ethoxy units a cyanopropyl column and a 95/5 acetonitrile/water mobile phase produced very good separations and peak shapes. Detection limits of 1 μg/L for individual surfactants were reported.

Thirty-one separate peaks were generated during the analysis of nonionic octylphenol polyether alcohol surfactants on a C_{18} column (photodiode array detector) (852). The use of an isocratic 25/75 acetonitrile/water mobile phase produced excellent peak shapes. The chromatogram exhibited two definite groups of peaks. The first 15-peak group eluted at 2–14 min and the second 16-peak group, at 18–80 min. No individual peak identification was given.

7.4.2 Polymeric Analytes

Polystyrene oligomers (n = 1–14) were separated according to their oligomer number and syndiotactic and isotactic isomers as well (757). The separation was accomplished using a two step 60-min gradient of (80/20 acetonitrile/water)/acetonitrile/dichloromethane (100/0/0–0/100/0 (at 30 min) to 0/50/50) on a C_{18} column (λ = 254 nm). The final oligomer eluted at 55 min.

Poly(ethylene glycols) (PEGs) of molecular weight 200–1560 were analyzed on a C_{18} column using various acetonitrile/water mobile phases (853). An interesting and ingenious departure from the norm was presented in this method: the addition of a visualizing reagent that deliberately generated a well-controlled nonzero background absorbance (i.e., indirect detection at λ = 210 nm). This precluded the need for the analyte of interest to have a chromophore or be derivatized prior to analysis since the visualizing reagent was displaced in proportion to the amount of analyte present and was monitored. However, both positive and negative peaks appear in the chro-

matogram, and so a data acquisition system capable of handling this is necessary. For PEG standards of molecular weight 200, 400, and 1000, the chromatographic conditions were as follows: 10/90 acetonitrile/water (0.8 mM caffeine), 210 nm, 15 min elution; 15/85 acetonitrile/water (0.8 mM *p*-hydroxybenzoate), 255 nm, 30 min; 20/80 acetonitrile/water (0.8 mM *p*-hydroxybenzoate), 255 nm, 30 min, respectively. This method separated up to the 35-mer in a PEG 1540 sample. Peak shapes were very good.

The anaerobic degradation products of PEGs were analyzed on a C_{18} column (λ = 190 nm) using a 41-min [(50/50 acetonitrile/water (1 mM H_3PO_4 + 3ppm HNO_3))]/water (1 mM H_3PO_4 + 3 ppm HNO_3) gradient (4/96–72/28) (854). This analysis was possible at 190 nm because the UV cutoff for acetonitrile is usually λ = 188 nm. The addition of nitric acid was a nifty trick used to artificially produce a background absorbance in the water so that the change in background absorbance during the gradient would be acceptable. The resulting chromatogram of a 50 mM mixture of PEGs with molecular weights 200, 300, 400, 600, 1000, and 1500 generated excellent peak shapes and profiles up to n = 50.

Residual monomers of epoxy resins (i.e., *m*-xylylenediamine and bisphenol A diglycidyl ether) were extracted from cured epoxy resins and analyzed on a C_{18} column (λ = 275 nm, ex; 300 nm, em for bisphenol; 395 nm, ex; 480 nm, em for *m*-xylylenediamine) using a complex 20-min acetontrile/water gradient (30/70–75/25) (855). Excellent resolution of bisphenol F, bisphenol A, three bisphenol F diglycidyl ether isomers, and bisphenol A diglycidyl ether resulted. Peak shapes were also excellent. Linear concentration curves from 20 to 1000 μg/L were obtained. The *m*-xylylenediamine was derivatized with fluorescamine and analyzed with the same column and gradient as for bisphenol (but with different excitation and emission wavelengths), and linear curves from 10 to 800 μg/L were obtained.

A series of three preservatives that release formaldehyde on degradation (Germall-115, Glydant, Dowicil-75) were resolved and separated from formaldehyde as their 3,5-diacetyl-1,4-dihydrolutidine derivatives (856). A PRP–1 column (λ = 410 nm) and a 30/70 acetonitrile/water mobile phase were used to generate the 15-min separation. Detection limits of 400 ppb and a linear range of two orders of magnitude were reported. The authors note that standards made from pure preservatives show substantial formaldehyde peaks upon analysis, i.e., in the short time between standard preparation and analysis, degradation occurs. No comment on the possible reason for the degradation (or source of contamination) was discussed.

The photochromic spirooxazine compound deposited in polyurethane films (i.e., 1,3-dihydro-1,3,3-trimethylspiro [2*H*-indole-2,3′]-[3*H*]naphth[2,1-*b*][1,4]-oxazine) was photodegraded and the products, substituted indole and naphthalene fragments, were monitored by HPLC (857). A C_{18} column (photodiode array detector, λ = 200 –450 nm) and a 40-min acetonitrile/water (40/60–100/0) gradient generated a baseline separation between the original spirooxazine and four identified degradation compounds.

Methyl methacrylate, *N*,*N*-dimethyl-*p*-toluidine, and benzoyl peroxide were extracted from various methyl methacrylate polymers and analyzed on a C_{18} column (λ = 235 nm) using a 52/48 acetonitrile/water mobile phase (858). Elution was

complete in 30 min. Bisphenol A and 4,4′-dichlorophenylsulfone were similarly extracted and resolved on a C_{18} column (λ = 235 nm) using a 50/50 acetonitrile/water mobile phase. Elution was complete in 22 min.

Residual isocyanate monomeric extracts from 19 commercial polyurethanes were analyzed as their 9-(methylaminomethyl)anthracene derivatives on a C_{18} column (λ = 254 nm, ex; 412 nm, em) at 45°C. An 80/20 acetonitrile/water (3% TEA to pH = 3 with H_3PO_4) mobile phase was used (859). Analytes included 2,4- and 2,6-toluenediisocyanate; 2,4-toluene diisocyanate dimer; diphenylmethane-4,4′-diisocyanate; hexamethylene diisocyanate; cyclohexyl-, phenyl-, and octadecylisocyanate; and isopherone diisocyanate. The effect of varying the level of acetonitrile (70–85%) on retention (as k') was presented as a plot for six different C_{18} materials. Detection limits were reported as 0.03 mg/kg.

7.4.3 Other Industrial Analytes

The analysis of fullerenes has received much recent attention and has been detailed in Chapters 3 and 4. Because fullerene is sparingly soluble in acetonitrile, it was not an effective solvent on its own. Consequently, it was used in conjunction with other solvents such as toluene (240,536) and dichloromethane (538,657). When too high a level of acetonitrile was present in the mobile phase, the fullerenes precipitated onto the column packing. The resolubilization process was controlled by the rate at which the mobile phase passes over the precipitate. Therefore, these samples often elute as severely tailed or double peaks due to the dual "retention" mechanism process: precipitation–solubilization followed by partition–adsorption.

Diesel oils were characterized by fractionating a sample into basic and neutral fractions and subsequently analyzing each fraction individually (860). The basic fraction was then analyzed for C_2–C_4 alkylbenzoquinone content and the neutral fraction for C_1–C_5 alkylcarbazole content. Both fractions were analyzed on a C_{18} column (particle-beam MS) using a 40-min acetonitrile/water gradient (40/60–100/0).

Gasoline was tested for kerosene adulteration by monitoring the naphthalene content (associated with kerosene) of the gasoline sample (861). The sample was prepared to be 1% v/v gasoline in methanol. An 80/20 acetonitrile/water mobile phase and a C_{18} column (λ = 285 nm) separated the analytes of interest: naphthalene, 2-methylnaphthalene and 2,6-dimethylnaphthalene. Elution was complete in < 15 min.

In an excellent study, White and Catterick (862) characterized and analyzed 40 acidic dyes according to their composition, shape, and retention on a PLRP-S column (photodiode array detector, λ = 190–600 nm). A 50/50 acetonitrile/water (0.7 g/L citric acid + 3.4 g/L tetrabutylammonium hydrogen sulfate adjusted to pH = 9.0 with ammonia solution) mobile phase was used. Five classes of dyes were included in the study: phenyl-N=N-monosulfonated naphthols, naphthyl-N=N-naphthols, phenyl-N=N-*p*-hydroxylated naphthyls, monosulfated phenyl-N=N-naphthols, and phenyl-N=N-disulfonated naphthols. Tables were generated in which the relative retention times, relative absorbances (vs. 500 nm) for 250–450 nm (50-nm intervals), and UV maximum absorbances were listed. From positional and spatial arguments

and data presented in the study, information about the chemical structure of an analyte could be determined with as little as 100 ng of sample. Structures were presented for all compounds.

Seventeen blue writing inks were characterized using a C_{18} column (photodiode array detector, λ = 200–800 nm) and an 80/20 acetonitrile/water (5 mM heptanesulfonic acid with acetic acid to pH = 4.7) mobile phase (863). Analysis was complete in 20 min. A set of retention versus wavelength versus intensity plots were given for different ink samples, and a number of ways of analyzing the data were presented. The authors noted that the acetonitrile/water mobile phase gave optimal results as compared with methanol/water mobile phases. Red printing inks were analyzed in a similar fashion using a C_{18} column (λ = 254, 510 and 350 nm, ex; 550 nm, em) and an acetonitrile/water (10 mM $KClO_4$ to pH = 3 with $HClO_4$) gradient (30/70–100/0) (242). Chromatographic profiles were presented and detection limits of ~50 ng reported.

Sixty-seven lots of Sunset Yellow FCF (FD&C Yellow No.6) were tested for combined benzidine on a C_{18} column (λ = 500 nm) using a 50-min acetonitrile/water (with 1.5% ammonium acetate and 0.5% acetonitrile) gradient (0/100–20/80) (864). Detection limits for combined benzidine were reported as 10 ng/g of sample.

Tris (2-chloroethyl) phosphate and six metabolites [e.g., bis(2-chloroethyl)hydrogen phosphate, bis(2-chloroethyl)2-hydroxyethyl phosphate, and its glucuronide analog] were extracted from urine and separated on a C_{18} column (^{14}C radioactivity detector) with a 28-min acetonitrile/water (0.1% TFA) gradient (865).

7.4.4 Summary

Acetonitrile has been widely used in the separation and quantitation of small industrial compounds. However, many polymers and large hydrocarbonaceous molecules have low solubility in acetonitrile, thereby limiting its use.

7.5 BIOLOGICAL ANALYTES

7.5.1 Carboxylic Acid Analytes

Eighteen carboxylic acids (e.g., caprylic, capric, lauric, myristic, palmitoleic, arachidonic, palmitic, oleic, stearic, gondoic) were separated as their 2-(2,3-anthracenedicarboximido)ethyl derivatives on a C_{18} column (λ = 298 nm, ex; 456 nm, em) at 50°C. A 45/135/20 acetonitrile/methanol/water mobile phase was used (866). Each peak was discernible, but a number were poorly resolved. The authors noted that the fluorescence intensity of these compounds was typically 15% greater in acetonitrile than in methanol. Linear concentration ranges of ~15 fmol-60 pmol and detection limits (S/N = 3) of 0.5–3 fmpol were found. Another study (867) used 1-pyrenyldiazomethane as a derivatizing reagent for carboxylic acids. Both short chain acids (lactic, formic, and propionic) and long chain acids ($C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, $C_{20:0}$,) were well resolved on a C_{18} column (λ = 340 nm, ex; 395 nm,

em) when a 50/50 acetonitrile/water isocratic mobile phase or a 60-min 85/15–100/0 acetonitrile/water gradient was used, respectively. Four derivatized prostaglandins (E_2, $F_{\alpha 2}$, E_1, $F_{\alpha 1}$) were also resolved using a 75/25 acetonitrile/water mobile phase. Detection limits were reported as 20–30 fmol injected.

Resolution of the 4,5-diaminophthalhydrazide derivatives of nine α-keto acids (e.g., α-ketobutyric, α-ketoisovaleric, *p*-hydroxyphenylpyruvic, α-ketocaproic, phenylpyruvic) was accomplished on a C_{18} column (chemiluminscence with hydrogen peroxide/potassium hexacyanoferrate solution) with a 13/87 acetonitrile/water (50 mM phosphate buffer pH = 7) (868) mobile phase. Elution was complete in 48 min. Detection limits of 4–50 fmol (10-μL injection) were reported.

Twenty-two carboxylic acids and carbonyl compounds (oxalacetic acid, glyoxylic acid, glyceraldehyde, glyoxal, acetone, isobutyraldehyde, benzaldehyde, cyclohexanone) were chromatographed as their 2,4-dinitrophenylhydrazine derivatives on a C_{18} column (λ = 360 nm) at 55°C. A complex (1/1.4/6 acetonitrile/methanol/water (5 mM KH_2PO_4 buffer pH = 6.7)/[(3/2 acetonitrile/water (5 mM KH_2PO_4 buffer pH = 6.7)] gradient (100/0–0/100) was used (869). In this study both mobile phase composition and flow rates were changed throughout the course of the separation. Complete resolution was not achieved between all 22 compounds, but the authors note that the presence of a significant level of methanol (~25%) was required to generate adequate resolution between most peaks. Detection limits from 15 to 500 ng/L were reported.

A series of phenacyl esters of fatty acids (C_6–C_{22}, even) were resolved on a C_{18} column using a 30-min acetonitrile/water gradient (70/30–100/0) (128). Interestingly, even though the compounds were baseline resolved and exhibited good peak shape, the use of methanol as the organic constituent in the mobile phase generated a more effective separation.

Benzoic acid extracted from orange juice was the subject of a collaborative study that used a PRP–1 column (λ = 230 nm) and a 40/60 acetonitrile/water (50 mM phosphate buffer pH = 2.3) mobile phase (870). Elution was complete in 8 min and a detection limit of 0.5 ppm (S/N = 10) was reported. The working range was 0.5–10 ppm.

Caffeic acid and caffeic acid *o*-quinone were resolved from 12 polyphenol oxidase products on a C_{18} column (λ = 280 nm) using a 40-min acetonitrile/water (2.5% acetic acid) gradient (4/96–36/74) (871). A working curve from 0.01 to 1.0 mM caffeic acid (20 μL injected) was used. The analytes of interest were baseline resolved from one another and from the other products.

Vanillylamine, L-phenylalanine, caffeic acid, coumaric acid, ferulic acid, cinnamic acid, capsaicin, and dihydrocapsaicin were extracted from green peppers and resolved on a C_{18} column (λ = 236 nm) using a 35-min acetonitrile/water (pH = 3.0, buffer not given) gradient (0/100–100/0) (887). The phenylalanine peak was severely tailed, but still did not coelute with other compounds of interest. The choice of a better buffer or a higher buffer concentration might decrease the tailing. No detection limits were given, but a table of results listed most analyte levels down to 50 μg/g.

A series of 24 prostaglandins (e.g., thromboxane, TX; leukotriene, LT; hydroxyeicosatetraenoic acid, HETE) were analyzed on a C_{18} column (λ = 192, 237, or

280 nm) using a 30-min acetonitrile/methanol/water/formic acid gradient (59/41/0/0.07–0/0/100/0.03) (919). The elution order for selected compounds was 20-COOH LTB_4 < TXB_2 < estradiol (3.5 min) < PGB_2 < 6-*trans*-LTB_4 < LTB_4 (7.5 min) < progesterone < 15-HETE < 11-HETE < 12-HETE (20 min) < LTC_4 < LTE_4 < LTD_4 (30 min). Peak shapes were excellent, and good resolution was obtained.

Seventeen hydroperoxy polyunsaturated fatty acids [e.g., 5-(*S*)-hydroperoxy-eicosatetraenoic acid, 5(*S*)-hydroperoxyeicosatetraenoic acid] were extracted from brain tissue and derivatized with acetic anhydride (874). The resulting solution was injected onto a C_{18} column (λ = 235 nm) and eluted with a 15/8/2 acetonitrile/water (0.1 M ammonium formate)/0.1 M formic acid mobile phase. The internal standard (12-hydroxyoctadecadienoic acid-d_8) was made up at the 600-pmol level and stored in a solution containing BHT to prevent its oxidation. Thermospray MS was also used as a detector and was most sensitive when a 15/6/4 acetonitrile/0.1 M acetic acid/0.1 M ammonium acetate mobile phase was used. In this example, detection limits of 0.1–0.5 pmol were obtained by single-ion monitoring (SIM).

A set of three chromatograms detailed the separation of short chain (C_4–C_8, methyl-C_3, methyl-C_4), medium-chain (C_5–C_8, C_{11}–C_{14}), and long-chain (C_{12}–C_{18}, even) fatty acids on a C_{18} column (λ = 205 nm) using three different parabolic acetonitrile/water gradients: 10/90–85/15, 20/80–100/0, and 70/30–100/0, respectively (875). Linear concentration curves from 60 to 800 nmol were obtained, and detection limits of ~50 nmol were reported.

Seven fatty acids ($C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{20:4}$,) were extracted from cardiac tissue and analyzed as their 9-anthryldiazomethane derivatives (876). Excellent peak shapes and baseline resolution were obtained on a C_{18} column (λ = 365 nm) using a 93/7 acetonitrile/water mobile phase. The total analysis time was 60 min. The authors noted that small changes in the mobile phase composition or minor deviations from a flow rate of 0.6 mL/min destroyed the resolution between the $C_{14:0}$, $C_{16:1}$, $C_{18:2}$, and $C_{20:4}$, compounds. The detection limits (S/N = 4) were reported as 15 fmol per 10-μL injection. Linear working curves are shown for up to 400 pmol injected.

Dicarboxylic acids (C_6–C_{16}, even) were analyzed as their monocoenzyme A, monocarnitine, and 4-nitrobenzyl esters (877). The monocoenzyme A esters were baseline resolved on a C_{18} column (photodiode array detector, λ = 200–300 nm) using a complex 45-min acetonitrile/water (50 mM KH_2PO_4 buffer pH = 5.3) gradient (5/95–50/50). Peak shapes were excellent. The monocarnitine esters were analyzed as their 4-bromophenacyl derivatives on a C_8 column (λ = 260 nm) using a complex 40-min acetonitrile/water/water (0.15 M triethylamine phosphate buffer pH = 5.6) gradient (60/38/2–95/0/5). The 4-nitrobenzyl derivatives were resolved on a C_{18} column (λ = 265 nm) using a complex 40-min acetontrile/water gradient (40/60–100/0). Peak shapes were excellent.

Arachidonic, palmitoleic, linoleic, eicosatrienoic, oleic, palmitic, and stearic acids were extracted from venous blood and baseline resolved as their 4-bromomethyl-7-methoxycouramrin derivatives in 40 min on a C_{18} column

(λ = 325 nm, ex; 398 nm, em) using an 85/15 acetonitrile/water mobile phase (878). Working curves were generated for concentrations of 50–500 μmol/L. Detection limits (S/N = 3) of ~10 μmol/L were reported (20 μL injected). Samples were diluted in solutions containing BHT to prevent oxidation of the acids.

Ten steryl ferulate and *p*-coumarate esters were extracted from corn and rice and were identified using a C_{18} column (λ = 325 nm) and an 82/3/2/13 acetonitrile/1 butanol/acetic acid/water mobile phase. Good resolution was obtained, and the elution was complete in 35 min. Chromatograms showing the effects of varying water level (1–21%) on peak shape and resolution were shown. Levels from 70 μg to 26 mg/g per sample were tabulated (879).

7.5.2 Basic Amine Analytes

Five polyamines (1,3-diaminopropane, putrescine, cadaverine, spermidine, and spermine) were extracted from freeze-dried plant and animal tissue and baseline resolved as their dansyl chloride derivatives. A C_{18} column (λ = 252 nm, ex; 500 nm, em) and an acetonitrile/water rapid gradient (70/30 for 4 min to 100/0 in 1 min) generated the separation (880). Detection limits of 0.5 pmol and a linear concentration range up to 35 pmol were reported. Elution was complete in $<$ 10 min. The dansyl derivatives of the preceding compounds and N^8-acetylspermidine were resolved on a C_{18} column (λ = 340 nm, ex; 510 nm, em) using a two-tier 5.5-min acetonitrile/water (10 mM heptanesulfonate at pH 3.4) gradient (40/60–100/0) (881). Excellent resolution and peaks shapes were achieved, and elution was complete in $<$ 8 min. Detection limits of 1 pmol injected and linear ranges of $<$ 200 pmol injected were reported.

Ten aromatic and aliphatic amines, including ammonia (!), (dimethyl-, diethyl-, 2-butyl-, *n*-butyl-, isobutyl-, benzyl-, diphenyl-, dibutyl- and decylamine) were separated as their phenyl isocyanate derivatives on a C_{18} column (λ = 240 nm) (882). A 20 min acetonitrile/water (20/80–70/30) gradient generated the separation. Peak shapes were excellent. Elution was complete in 22 min. Detection limits of 1 ng injected were reported.

The urinary metabolites of 5-hydroxyindole (5-hydroxytryptophan, 5-hydroxytryptamine, 5-hydroxyindol-3-ylacetic acid, 5-hydroxytryptophol, *N*-acetyl-5-hydroxytryptamine) were baseline resolved on a C_{18} column using a 5/95 acetonitrile/water (10 mM acetate buffer pH = 4.7) mobile phase (883). The compounds were postcolumn-derivatized with a benzylamine/potassium hexacyanoferrate(III) mixture (λ = 345 nm, ex; 481 nm, em). Elution was complete in 40 min. Detection limits (S/N = 3) were reported in the range of 100–500 fmol injected (analyte-dependent). Peak shapes were excellent.

An optimization procedure for the separation of epinephrine bitartrate, L-dopa, 3,4-dihydroxyphenylacetic acid, norepinephrine · HCl, and dopamine · HCl (with 3,4-dihydroxybenzylamine · HCl as internal standard), was described by He et al. (884). A C_{18} column was used in conjunction with an electrochemical detector (+0.6 V vs. Ag/AgCl). A window diagram of relative retention times for adjacent eluting solute pairs (i.e., t_{R2}/t_{R1}) resulted in three acceptable solvent composition windows.

The optimal solvent conditions were found to be 2.5/97.5 acetonitrile/water (0.23% sodium acetate + 0.02% EDTA + 0.066% sodium heptanesulfonate adjusted to pH = 3.9 with monochloroacetic acid). Elution was complete in < 30 min, and all peaks were well resolved. Detection limits for canine or human plasma samples were reported as ~10 pg/mL for epinephrine and norepinephrine.

Seventeen monoamine neurotransmitters were well resolved in an excellent method developed by Wester et al. (885). Vanillylmandelic acid, metanephrine, 3-methoxy-4-hydroxyphenylethylene glycol, isoproterenol, serotonin, epinephrine, 5-hydroyindoleacetic acid, 3-methoxytyramine, and homovanillic acid were included in the analyte set. Elution was complete in 22 min on a C_{18} column (electrochemical detector at +0.2 V and +0.8 V) using a 6.3/93.7 acetonitrile/water (0.1 M citrate buffer + 0.3 mM sodium EDTA + 0.3 mM sodium octyl sulfate pH = 2.35) mobile phase. Injections of 15 pmol of analyte were readily detected. In optimizing the separation, the effects of changing the concentrations of acetonitrile, octyl sulfonate, and EDTA as well as the pH and temperature were tabulated for each analyte.

Serotonin (and 5-hydroxyindoleacetic acid) and a total of eight catecholamines and metabolites including norepinephrine and dopamine (and their major metabolites, e.g., 4-hydroxy-3-methoxyphenylethylene glycol, homovanillic acid) were separated from epinephrine, tyrosine, and tryptophan on a C_{18} column (λ = 230 nm ex; 320 nm em, or amperometric detection at +0.7 V vs. Ag/AgCl on glassy carbon) at 30°C using a 19/181 acetonitrile/water (12.16 mM citric acid, 11.60 mM diammonium phosphate, 2.54 mM sodium octyl sulfate, 3.32 mM dibutylamine phosphate, 1.11 mM sodium EDTA) mobile phase (886). Standards and samples were stored at −70°C and used for only one day. Brain tissue extracts were studied and detection limits of 75 pg injected (17 ng/g) for tyrosine to 520 pg injected (120 ng/g) for tryptophan were reported for fluorescence detection. 5-Homovanillic acid and 3,4-dihydroxyphenylacetic acid were not detected by this method. With amperometric detection, all analytes gave a response, and the detection limits ranged from 25 pg injected (6 ng/g) for dopamine and 50 pg injected (12 ng/g) for 3-methoxytyramine. Elution of all components was complete in 20 min, and resulting peaks shapes were excellent.

Thymine and six photoirradiation products generated in the presence of *p*-aminobenzoic acid (5,6-dihydrothymine, 5-hydroxymethyluracil, *cis-anti-*, *cis–syn-*, *trans–anti-*, and *trans–syn*–cyclobutane dimers of thymine) were separated on a C_{18} column (photodiode array detector, λ = 200–400 nm) using a 30-min acetonitrile/water (75 mM phosphate buffer pH = 4.4) gradient (0/100–50/50) (887). Elution was complete in 25 min, and good peak shapes were obtained.

Caffeine and four metabolites—theobromine, paraxanthine, theophylline, and 1,3,7-trimethyluric acid—were extracted from liver tissue and resolved on a C_{18} column (λ = 250 nm) using a 30-min (895/95/7/3 water/acetonitrile/THF/acetic acid)/(695/295/7/3 water/acetonitrile/THF/acetic acid) gradient (85/15–5/95) (888). Samples were reconstituted in the initial mobile phase, and phenacetin and 4-hydroxyanilide were also added. Resolution and peak shapes were excellent, and elution was complete in 55 min.

Ten pair of racemic nicotine analogs [e.g., *N'*-(2,2-difluoroethyl)nornicotine, *N*-benzyl-*N*-dimethyl-3-pyridinemethananime, 1-benzyl-2-phenylpyrrolidine, *N'*-(2-methylbenzenyl)nornicotine, 5,6-cyclohexenonicotine] were individually resolved on a β-cyclodextrin column (λ = 254 nm) using various isocratic acetonitrile/water (1% TEA to pH = 7.1 with acetic acid) mobile phases (889). The mobile-phase composition (ranging from 5/95 to 30/70), resulting *k'* values, and separation factors (α) were tabulated for each pair of compounds. A 20-min gradient of acetonitrile/water (buffer as above) from 10/90–70/30 led to the baseline resolution of three racemic analyte pairs: *N'*-benzylnornicotine, *N'*-(2,2,2-trifluoroethyl)nornicotine, and *N'*-(2-naphthylmethyl)nornicotine. A series of 5–10-μL injections of 0.1–0.5% samples were used. An interesting study of the retention of racemic *N'*-benzylnornicotine in both methanol and acetonitrile/buffer mobile phases (both organic levels at 20–100%) was presented. For methanol, *k'* decreased, as expected, with increasing % methanol. For acetonitrile, however, the *k'* decreased to 40% acetonitrile, stayed constant to 80% acetonitrile, and then increased from 80 to 100% acetonitrile. The authors speculate that the retention increase was caused by either increased hydrogen-bond interaction with the surface or another surface complexation process. The former explanation was consistent with the differences between acetonitrile (weak hydrogen-bond acceptor) and methanol (strong hydrogen-bond acceptor and donor).

An excellent separation of 14 heterocyclic aromatic amines (e.g., aminoimidazoazaarenes such as 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; glutamic acid pyrolysates such as 2-amino-6-methyldipyrido[1,2-a:3′2′-d]imidazole; carbolines such as 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*f*]indole) extracted from fried or broiled meats was developed on a C_{18} column (λ = 263 nm and fluorescence with excitation/emission wavelength pairs specific to each compound) (890). A complex 55-min acetonitrile/water (10 mM TEA adjusted to pH = 3.2 with H_3PO_4)/water (10 mM TEA adjusted to pH = 3.6 with H_3PO_4) gradient (5/95/0– 15/85/0–15/0/85–55/0/45) was used. The analytes of interest eluted in < 30 min, peak shapes were uniformly excellent, and detection limits of "low ng/g" were reported.

Five biotransformation products of *N*-methylbenzylamidine (benzamide, benzamidine, *N*-methylbenzamide, *N*-hydroxy-*N*-methylbenzamidine, and *N*-methylbenzamidoxime) were baseline resolved in < 30 min on a C_{18} column (λ = 229 nm) using 12/88 acetonitrile/water (0.08% 85% H_3PO_4 and 0.001% sodium octanesulfonate). Peak shapes were very good, and a working curve of 5–300 pmol injected was reported (891).

Putrescine, cadaverine, spermidine, and spermine were extracted from plant materials and derivatized with 9-fluorenylmethyl chloroformate (FMOC) (892). Separation was achieved on a C_8 column (λ = 265 nm, ex; 310 nm, em) using a complex 35-min [20/0.5/79.5 acetonitrile/THF/water (0.1 M sodium acetate buffer pH = 4.2)]/[79.5/0.5/20 acetonitrile/THF/water (0.1 M sodium acetate buffer pH = 4.2)] gradient (50/50–100/0). These compounds were well resolved from other compounds that reacted with the FMOC reagent, such as methanol, aspartic acid (used to react with the excess FMOC reagent), and ornithine. Peak shapes were good, and detection limits were reported at 7–28 pg (analyte-dependent).

7.5.3 Aflatoxins, Mycotoxins, and Other Toxic Analytes

The classic separation of aflatoxins B_1, B_2, G_1, and G_2 was reviewed by Shepherd (281) and Gilbert (893). Often isocratic acetonitrile/water or acetonitrile/methanol/water mobile phases are used in conjunction with C_{18} columns (λ = 360 nm). Normally acetonitrile levels of 25–40% are used. If methanol is used, it is approximately at the 5–15% level and directly replaces an equivalent amount of acetonitrile. Most importantly, TFA is almost invariably added at the 0.1–0.5% level. TFA forms a hemiacetal product with the aflatoxins, which increases sensitivity by a factor of $\geq$ 3. Complete baseline resolution is usually achieved in $<$ 20 min. Fluorescence detection has been used with a 360-nm excitation and a 440-nm emission wavelength chosen. Detection limits of 1–10 pg were commonly reported. Postcolumn derivatization with iodine has also been used in conjunction with fluorescence detection to increase sensitivity.

Ten microcystins (algal hepatotoxins) were extracted from water samples and separated on a C_{18} column (photodiode array detector, λ = 200 − 300 nm) using a complex 50-min acetonitrile/water (0.05% TFA) gradient (30/70–0/100). Excellent peak shapes and baseline resolution for all analytes were obtained. The microcystins included in the study were microcystin-RR, -YR, -LR, -FR, -LA, -LY, -LW, -LF, and nodularin. Working-curve concentration ranges of 34 ng–9 μg/L were used (894).

Murata et al. (895), separated three microcystins (RR, YR, and LR) as the dansylated derivatives. Peroxyoxalate chemiluminescence detection was chosen using a 0.5 mM bis[4-nitro-2-(3,6,9-trioxadesyloxycarbonyl)phenyl] oxalate + 50 mM hydrogen peroxide postcolumn reaction. This method gave a detection limit (S/N = 10) of 15 fmol. Linear working ranges were reported as 15–1670 fmol. Separation was achieved on a C_{18} column (Jasco 885-CL chemiluminescence detector was optimal) with a 40/60 acetonitrile/water (0.05% TFA) mobile phase. Peak shapes and resolution were excellent, and elution was complete in $<$ 10 min.

In an excellent study seven mycotoxins (deoxynivalenol, patulin, diacetoxyscirpenol, HT–2 toxin, T–2 toxin, zearalenone, ochratoxin A) were baseline resolved on a C_{18} column (photodiode array detector, λ = 210 − 340 nm) at 35°C using a 7-min acetonitrile/water (phosphoric acid to pH = 3) gradient (40/60–70/30) (896). Peak shapes were excellent, and detection limits of 5–20 μg/20 g wheat were reported. Linear concentration curves from 40 to 800 ng injected were obtained. The UV spectra for all analytes in a 30/40 acetonitrile/water were presented.

Altersolanols A, B, C, D, E, and F were extracted from *Alternaria solani* and baseline resolved on a C_{18} column (λ = 270 nm) at 25°C using a complex 23-min acetonitrile/water gradient (18/82–90/10) (897). Excellent peak shapes were generated. Detection limits (S/N = 3) of 0.2–0.5 pmol 5 μL injection were reported. A limited linear working range of 0.4–1.0 μM was also reported.

Hederasaponins B, C, and D and α-, β-, and γ-hederin were extracted from leaves and baseline resolved on a C_{18} column (ELSD) using a 25-min acetonitrile/water gradient (35/65–63/37). The last analyte of interest eluted in 20 min. Peak shapes were excellent, and detection limits of $\sim$ 0.1 μg/20 μL were reported (898).

Thirteen free and conjugated bile acids (e.g., cholate, taurocholate, deoxycholate, lithocholate, taurolithocholate, glycodeoxycholate, all as sodium salts) were baseline resolved on a PLRP-S column (pulsed amperometric detector at +0.03 V vs. Ag/AgCl) at 35°C (899). A 55-min acetonitrile/water/0.5 M aqueous NaOH (20/60/30–29/51/20) gradient was used. The presence of 0.1 M hydroxide is essential for the amperometric detection of the hydroxy functional group on the bile acids. However, the authors note that acetonitrile slowly decomposes to ammonium and acetate ion under these basic conditions. Therefore, postcolumn addition of the hydroxide is recommended. Peak shapes were excellent, and detection limits (S/N = 3) of 60 pmol injected were reported. A linear concentration range of 0.6–14 μmol (100-μL injection) was obtained.

Seven of the major bile acids (e.g., taurocholate, α- and ω-tauromuricholate, glycohyodeoxycholate) were extracted from bile and serum and analyzed on a C_{18} column (λ = 200 nm and FAB-MS) using a complex 45-min (97/3 acetonitrile/glycerol)/(95/5 water (19 mM ammonium carbonate buffer pH = 4.0)/glycerol) gradient (20/80–90/10) (900). Detection limits for the UV work was reported as 0.1 nmol. Peak shapes were good, and one pair coeluted (taurocholate and taurohyodeoxycholate) but were successfully monitored by MS.

Iida et al. (901) studied the separation of 15 free, glycine-, and taurine-aminated bile acids (lithocholic, chenodeoxycholic, ursodeoxycholic, deoxycholic, and cholic acids) on a C_{18} column (λ = 205 nm). A number of acetonitrile/water (phosphate buffer pH = 3.5–7.5) mobile phase combinations were examined. The optimal resolution occurred with a 23/77 acetonitrile/water (0.3% phosphate buffer pH = 7.0) mobile phase. Eleven peaks eluted in $<$ 40 min, but four sets of peaks coeluted. The remaining peaks were eluted in 20 min using a 28/72 acetonitrile/water (0.3% phosphate buffer pH = 7.0) mobile phase. On the basis of these results, a gradient starting at a lower percent acetonitrile and ramping to a higher percent acetonitrile finish would be particularly effective at increasing resolution while keeping analysis times reasonable.

7.5.4 Vitamins and Related Analytes

In a wonderfully short and effective separation, seven water-soluble vitamins (ascorbic acid, niacin, niacinamide, pyridoxine, folic acid, thiamine, riboflavin) were baseline resolved on a C_8 column using an isocratic 7/93 acetonitrile/water (1% acetic acid and 5 mM sodium heptanesulfonate) mobile phase (293). Elution was complete in 6 min.

Vitamins D_2 and D_3 were extracted from milk and were determined using a C_{18} column (λ = 265 nm) and a 97/3 acetonitrile/methanol mobile phase (902). They were fully resolved and eluted in $<$ 25 min. The authors noted that a 10-min reequilibration time with 97/3 acetonitrile/methanol was needed to generate reproducible results. Whether this was due to late eluting peaks or some other system variable was not indicated. Detection limits of 2.5 ng injected and working-curve concentrations of $\leq$ 60 ng were reported.

The retention behavior of glucuronides and sulfates of provitamin D, vitamins D_2, D_3, and 25(OH)D_3 as their 4-[4-(6-methoxy-2-benzoxazolyl)phenyl]-1,2,4-trazoline-3,5-dione derivatives was studied using a C_8 column (λ = 265 or 320 nm, ex; 380 nm, em) (903). The two sulfate conjugates of provitamin D were resolved in 10 min on a C_8 column using a 66/34 acetonitrile/water (2% $NaClO_4$ and 5 mM methyl-β-cyclodextrin) while the two glucuronide conjugates were resolved and eluted in 14 min under the same conditions. Detection limits (S/N = 5) of 5 fmol were reported. The identical conditions produced baseline resolution of the sulfate and glucuronide conjugates of vitamin D as well. Addition of cyclodextrin was critical to the separation. Without it, the sulfate conjugate peaks coeluted. Three positional isomers of vitamin 25(OH)D_3 glucuronides were baseline resolved in 15 min using a 50/50 acetonitrile/water (2% $NaClO_4$ to pH = 3.0 with $HClO_4$) mobile phase.

Andrikopoulos et al. (904) developed an excellent separation scheme for an interesting mixture of compounds: phenolic antioxidants, tocopherols, and trigycerides. This was accomplished on a C_{18} column (λ = 280 nm or photodiode array detector, λ = 210 − 330 nm) with a complex 67-min [water (H_3PO_4 to pH = 3.0)]/(7/5 acetonitrile/methanol)/IPA gradient (70/30/0→0/100/0→0/40/60). The 10 antioxidants (e.g., BHT, BHA, propyldodecylgallate, *t*-butylhydroquinone, nordihydroguaiaretic acid) were baseline resolved and completely eluted in the first 20 min. Tocopherols (α-, β-, γ-, and δ-) were baseline resolved and eluted between 28 and 34 min. Triglycerides with equivalent carbon numbers from 42 to 50 were eluted between 40 and 50 min. Differentiation of triglycerides was achieved through varying the detector wavelength: 215 nm for saturated and unsaturated triglycerides, 280 nm for conjugated triglycerides and 230 nm for oxidized unsaturated triglycerides. Individual 20-μL aliquots of working solutions containing 300 mg/mL triglycerides, 50 μg/mL antioxidants, and 300 μg/mL tocopherols were injected. Excellent peak shapes were obtained for all compounds.

Retinoids are routinely separated and quantitated using RP-HPLC (905). Most frequently a C_{18} column was used in conjunction with a UV detector, an acetonitrile/buffer mobile phase and wavelengths between 340 and 365 nm. Multiple retinoids were separated isocratically with acetonitrile/water (buffer such as 10 mM ammonium acetate) mobile phases, with the acetonitrile level ranging between 50 and 80%. Complex mixtures have required a gradient approach, and in these cases methanol has been frequently used as acetonitrile. A wide range of detection limits has been reported, but they are typically between 2 and 50 ng/mL.

Lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene, and β-carotene were extracted from fruits and vegetables and baseline resolved on a C_{18} column (λ = 450 nm) using a 75/20/5 acetonitrile/methanol/dichloromethane (0.1% BHT and 0.05% TEA) mobile phase (906). The authors note that carotenoids degrade on silica-based columns. The addition of TEA was found to stop the degradation process. For example, the average recovery of test analytes increased from 68 to 92% on the addition of 0.1% TEA to the mobile phase. Linear concentration curves from 0.05 to 4 μg/L and detection limits of ~0.02 μg/mL were reported.

Khachik et al. (907) studied the effect of injection solvents on the peak shapes of carotenoids. The study was conducted on a C_{18} column (λ = 450 nm) and a 30-min

acetonitrile / methanol / dichloromethane / hexane gradient (10/85/2.5/2.5–10/50/20/20) was used. When the sample was dissolved in either THF, dichloromethane or toluene double or multiple peaks were formed. Increased injection volumes exacerbated the problem. Most likely, this finding is the result of the extremely limited solubility the sample solvent has in the initial mobile phase. The retention process therefore is not partitioning but is a kinetically controlled de-solvation process. Acetonitrile, acetone, and methanol provided excellent peak shapes (they are all miscible with the initial mobile phase).

7.5.5 Terpenoids, Flavanoids, Steroids, and Related Analytes

Wolfender et al. (908) screened plant extracts for various phenolic and terpene glycosides (e.g., phlominol, sesamoside, lisianthioside, bellidifolin-8-*O*-glucoside) using an acetonitrile/water (0.05% TFA) gradient with C_8 or C_{18} columns (thermospray MS). The gradients were typically run from ~5–75% acetonitrile and lasted 30–50 min. (Note that ammonium ion for the MS process was added to the effluent as a postcolumn additive.) The same group also studied saponins (e.g., glucose, rhamnose, arabinose, glucuronic acid, glucosamine) from plants using the thermospray MS as the LC detector (909). In this case, a 30 min 30/70–80/20 acetonitrile/water gradient on a C_{18} column (λ = 206 nm) was used for generating the separation. The system effluent was made MS-compatible through the postcolumn addition of 0.5M ammonium ion. Injections of 2 μg of pure sample were used.

Seven constituents extracted from a Wuu-Ji-San preparation (liquiritin, hesperidin, cinnamic acid, cinnamaldehyde, glycyrrhizin, honokiol and magnolol) were baseline resolved on a C_{18} column (λ = 254 nm) using a 30-min acetonitrile/water (0.03% H_3PO_4) gradient (5/95–70/30) followed by a 20-min hold (910). Working concentration ranges from 5 ng to 125 μg/mL were used. Coelution of interferences was negligible, and peak shapes were excellent.

Six flavanone glycosides (eriocitrin, narirutin, hesperidin, neoeriocitrin, naringin, neohesperiden) were extracted from citrus juices and resolved on a C_{18} column (λ = 280 nm) (911). Elution was complete in 30 min when a 16/3/80/1 acetonitrile/THF/water/acetic acid mobile phase was used.

Theogallin, gallic acid, epicatechin gallate, catechin, caffeine, epicatechin, epigallocatechin gallate, and epigallocatechin were extracted from tea and separated on a C_{18} column (λ = 278 nm) using a 157/40/2/1 water/dimethyl formamide/methanol/acetic acid mobile phase (740). Baseline resolution was achieved, and elution was complete in 25 min.

The procyanidins extracted from grape seeds (many the same as those in the previous study) were separated by RP-HPLC (912). The initial extract was separated on a Sephadex LH-20 column into three fractions that were further separated on a C_{18} column (λ = 280 nm) using a complex 80-min acetonitrile (4.5% formic acid)/water (4.5% formic acid) gradient (0/100–100/0). Classification of each peak was attempted via retention time and UV spectra comparison for known compounds. This was an extremely informative paper for researchers doing work with this class of compounds. A table of retention times and compound identification was generated.

Anthocyanin pigment UV spectra were used to characterize and identify various plant extracts (913). A C_{18} column (λ = 520 nm for peak monitoring and photodiode array detector, λ = 250–610 nm for peak characterization) was used with an 85/15 acetonitrile/(85/15 water/acetic acid) acid mobile phase to generate the elution profiles for pepper, eggplant, blueberry, and huckleberry extracts. Elution was complete in 60 min. A second elution protocol was used to separate and identify 12 anthocyanins in huckleberry juice. Here a complex 40-min acetonitrile/water (4% H_3PO_4) gradient (12/78–25/75) was used in conjunction with the C_{18} column. The set of compounds identified included delphinidin-3-rutinoside-5-glucoside, petunidin-3-*p*-coumaroyl-rutinoside-5-glucoside, and malvidin-3-feroylrutinoside-5-glucoside.

Anthocyanins were also identified by LC-MS using a C_{18} column (liquid secondary ion MS and λ = 300 nm) (914) and a complex 40-min acetonitrile/water (5% acetic acid) gradient (0/100–100/0). In this fashion the major anthocyanin of *T. pallida* was determined to be cyanidin–3,7,3′-triglucoside. Hydrosylates of the extract were also characterized in order to assist in the identification process. Six hydrosylate peaks were well resolved using the separation protocol cited above.

Seven flavanoids (quercetin, pinobanksin, kaempferol, pinocembrin, chrysin, galangin,and tectochrysin) were extracted from sunflower honey and resolved on a C_{18} column (λ = 366 nm) (915) using a complex 60-min acetonitrile/water (phosphoric acid pH = 2.6) gradient (0/100–70/30). All compounds eluted between 40 and 55 min. Peak shapes were uniformly good, and interferent peaks were not a major problem.

Extracts of flavanoids from *Ginkgo biloba* were fingerprinted using a C_{18} column (λ = 350 nm) and a complex 30-min (65/35 THF/IPA)/acetonitrile/water (0.5% H_3PO_4) gradient (15/1.5/83.5–0/78/22) (329). Excellent separation of 33 compounds resulted with all peaks identified (e.g., quercetin, kaempferol, myricetin, isorhamnetin, luteolin, and their glycosylated and rhamnosylated analogs, apigenin, gingketin, and sciadopitysin). Peak shapes were uniformly excellent. In a similar fashion (732), 13 flavanol glycosides (e.g., myricetin-, kaempferol-, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside and quercetin-3-*O*-rhamnoside) were well resolved on a C_{18} column (λ = 354 nm) using an 85/15 acetonitrile/water (20 mL acetic acid/L) mobile phase. Elution was complete in 40 min.

Twenty nonvolatile constituents of black tea [e.g., theogallin, gallic acid, caffeine, *p*-coumaroylquinic acid, theobromine, (−)-epicatechin gallate, quercetin 3-glucoside, rhamnosylglucoside, theaflavine-3,3′-digallate] were resolved on a C_{18} column (photodiode array detector, λ = 220–600 nm) using a 50-min acetonitrile/water (2% acetic acid) gradient (8/92–31/69). Peak identification through matching spectra with literature-reported spectra were discussed for many analytes. Peak shapes were good (916). This work was expanded by the same group (917) with special emphasis on the classification of various peaks as belonging to specific chemical classes of materials (e.g., theaflavins, theaflavic acid, thearubigins). These separations were carried out as described earlier. However, other columns and elution conditions were also studied: a C_8 column with a 50-min acetonitrile/water (2% acetic acid) gradient (0/100–26/74) or a PRP-1 column with a 50-min acetonitrile/water gradient (5/95–33/67).

Sheu and Chen (918) baseline resolved eight extracted constituents of tea (e.g., 10,10′:threosennoside B, emodin, wogonin, orloxylin A 7-*O*-glucuronide, glycyrrhizin, baicalein) using a C_{18} column (λ = 254 nm) and a complex 50-min (8/1/1 water/acetonitrile/methanol (3.0 mM tetrabutylammonium phosphate + 7.3mM KH_2PO_4 adjusted to pH = 4.2 with H_3PO_4)/[(1/2/2 water/acetonitrile/methanol (3.0 mM tetrabutylammonium phosphate + 7.3 mM KH_2PO_4 adjusted to pH = 4.2 with H_3PO_4)] gradient (95/5–1/100). Plots of k' versus both KH_2PO_4 and tetrabutylammonium concentration were shown to be complicated and neither linear nor logarithmic in nature. A linear working range of 0.5–225 μg/mL (analyte-dependent) was reported for these compounds. Detection limits (S/N = 3) of 0.08–0.5 ng injected were obtained.

Szepesi and Gazdag (919) studied the retention of 22 eburnane alkaloids [e.g., (+)-*cis*-vincamine, (+)-*cis*-epivincaminic acid ethyl ester, (+)-*cis*-isovincanole, (+)-*cis*-10-bromovincamine, (+)-*cis*-apovincamine, (+)-*cis*-dehydroepivincamine] on C_8 and C_{18} columns (λ = 280 nm). The k' values for these compounds were tabulated for a series of isocratic acetonitrile/water [10 mM $(NH_4)_2CO_3$] mobile phases varying from 40/60 to 80/20 on the C_{18} column and from 40/60 to 70/30 on the C_8 column. A reasonable separation of all components was achieved on the C_{18} column using a 60/40 acetonitrile/water [10 mM $(NH_4)_2CO_3$] mobile phase. Elution was complete in 35 min. The mobile phase will be quite basic, and so a precolumn should be used to presaturate the mobile phase prior to reaching the analytical column.

Eight isoquinoline alkaloids (e.g., chelidonine, berberine, coptisine, dihydrosanguinarine) were extracted from *Chelidonium majus* and resolved on a C_{18} column (λ = 290 nm) using a 46/10/44 acetonitrile/methanol/water (0.05M tartaric acid and 0.5% sodium dodecylsulfate) mobile phase (920). Peaks were slightly tailed. Elution was complete in 40 min. Standards of 0.01–0.28mg /mL were used and 1-μL aliquots were injected. Capacity factors for all compounds were presented and plotted against percent acetonitrile (43–47%) over which range the average capacity factor deceased by ~40%.

Saponins extracted from the Phytolaccaceae plant were analyzed as both their underivatized (λ = 206 nm) and 4-nitrophenacyl derivatives (λ = 254 nm) on a C_{18} column. The underivatized compounds were eluted with a 60-min acetonitrile/water gradient (30/70–50/50) (921). Derivatized saponins were eluted with a 40-min acetonitrile/water gradient (40/60–70/30).

Cholesterol and nine 10-cholesteryl esters (e.g., linolenate, laurate, myristate, stearate, and arachidate) extracted from blood were separated on a C_{18} column (λ = 206 nm) using a 60/40 acetonitrile/IPA mobile phase (922). Peaks were somewhat broad, but overall resolution was good. The addition of more acetonitrile sharpened the peaks, but resolution suffered when the acetonitrile level went above 60%. The authors note that UV detection became impossible under 205 nm (due to IPA background absorbance) and that special testing of the IPA was necessary prior to use to ensure that the background absorbance would be acceptable. A linear working range of 0.3–50 μg injected (in 20 μL) was reported.

7.5.6 Analytes from Fats and Oils

The kinetics of lipoxygenase were followed by the relative change in amount of linoleic acid, 1-palmitoyl-2-linoleoyl-*sn*-glycerol-3-phosphoethanolamine, 1-linoleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine, and trilinolein in the sample (923). These compounds were baseline resolved on a C_{18} column (λ = 205 nm) using a complex 13-min acetonitrile/methanol/water gradient (0/86/14–51/48/1). Elution of the most retained peak, trilinolein, was at 21 min. As an alternative detection scheme, the authors note that ammonium acetate is compatible with an ELSD whereas choline chloride produced significant baseline noise.

The retention behavior of phosphatidic acid methyl esters ($C_{16}-C_{20}$) was studied on a C_{18} column (λ = 208 nm) using a 70/22/8 acetonitrile/methanol/water (5 mM tetraalkyltriethylammonium phosphate) mobile phase (924). A number of quaternary alkyltriethylammonium phosphates (pentyl, hexyl, heptyl, octyl, and dodecyl) were tested as the mobile-phase modifier. The pentyl gave the best separation. Capacity factors were tabulated for all analytes in all mobile phases. This method was presented as an alternative to the more common NP separations detailed in Chapters 3 and 4. Peaks were not well resolved indicating that a gradient may have given better results.

The phospholipids phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SPH) were baseline resolved on an aminopropyl column (λ = 205 nm) using a 61.2/28.9/10 acetonitrile/methanol/water [10 mM $(NH_4)H_2PO_4$ buffer pH = 4.8] mobile phase (925). Good peak shapes resulted, and elution was complete in 45 min. Diphosphatidylglycerol (DPG) can also be monitored using a 61.6/26.4/12 acetonitrile/methanol/water [10 mM $(NH_4)H_2PO_4$ buffer pH = 5.8] mobile phase. In this instance, all other phospholipid peaks were eluted in $<$ 20 min, with a concomitant loss of resolution, and the DPG was eluted in 40 min. Since no gradient was used, significant time was saved since no re-equilibration is necessary between injections.

Ramesha et al. (926) described the subclass separation of diacyl, alkylacyl and alkenylacylethanolamine phospholipids from brain tissues using the 1-anthroyl derivatives. A C_{18} column (λ = 254 nm) and a 70/30 acetonitrile/IPA mobile phase were used to generate the separation. Twenty-one peaks were generated and characterized as to their molecular species identity. The authors reported a detection limit (S/N=5) of 0.5 pmol injected for individual components and 2 pmol injected for mixtures. Linear concentration ranges up to 200 pmol injected were obtained.

A number of triacylglyceride separations were presented earlier using C_{18} columns and acetone/acetonitrile (761,762,766,768), propionitrile/M*t*BE (734), and acetonitrile/chloroform (927) mobile phases. Other methods using acetonitrile have been developed for TAGs and substituted TAGs.

Hydroxylated triacylglycerols from castor oil and beef tallow were separated according to their equivalent carbon number (ECN) using a C_{18} column (RI) at 35°C and a 74/26 acetonitrile/THF mobile phase (928). A 20-μL aliquot of a 10–20% TAG solution was injected. The ECN range went from 34 (retention time 3 min for

ricinoleoyl/ricinoleoyl/ricinoleoyl (RRR)) to 48 (retention time 45 min for stearoyl/stearoyl/stearoyl (SSS)) with palmitoyl, linoleoyl, hydroxystearoyl and juniperoyl substituents represented as well.

Kuksis et al. (929) studied the separation of short chain TAGs in butter oil and long chain TAGs in menhaden oil using a C_{18} column (negative chemical ionization direct inlet MS) and a 40-min acetonitrile/propionitrile (1% dichloromethane) gradient (10/90–90/10). The dichloromethane was added to produce a chloride attachment source that allowed for the unambiguous identification of the TAGs. The same group identified, quantitated (as a percent of the total sample), and tabulated the results for over 150 component peaks in the butter oil sample (930). An RP-HPLC method using the C_{18} column (ELSD) and a 40- or 90 min acetonitrile/IPA gradient (10/90–90/10) is also presented (931). These studies give valuable reference information for triacylglyceride separation and identification.

A synthetic mixture of TAGs ranging from tricaprylic to tristearic was resolved on a C_{18} column (RI) at 40°C using a unique isocratic mobile phase: 80/20 propionitrile/butyronitrile (932). Although propionitrile had been shown to be effective in the separation of TAGs (preceding references), it was found to have limited ability to solubilize the higher-molecular-weight TAGs. Therefore, the butyronitrile was used as the strong solvent in this study. Elution was complete in 60 min (with a very slow flow rate of 0.3 mL/min). Injections of 50 μg generated a good detector response.

7.5.7 Nucleotides, Nucleosides, and Related Analytes

Adenosine, inosine, hypoxanthine, xanthine, adenine, guanosine, and β-NAD^+ were extracted from placental tissue and baseline resolved on a C_{18} column (λ = 254 nm) using a complex 12-min acetonitrile/water (7.5 mM ammonium phosphate buffer pH = 6.0) gradient (0/100–20/80). ATP, ADP, AMP, and NADP were also resolved using this method (933). The separation of guanosine from hypoxanthine was incomplete in this mobile phase. Standards containing 50–200 pmol/20 μL of each analyte generated easily detected peaks and good peak shapes. Detection limits of low-nanomole-range analyte per gram tissue were reported.

The retention behavior of the 2′-deoxyribonucleosides (adenosine, cytidine, uridine, thymidine, guanosine) were studied on a C_{18} column (λ = 254 nm) with a series of mobile phases ranging from 100% water to 12.5/87.5 acetonitrile/water (934). Capacity factors for each compound were plotted and ranged from 5–90 in 100% water to 0.05–0.7 in 12.5/87.5 acetonitrile/water.

Ally and Park (935) analyzed creatine, phosphocreatine, four purine bases (e.g., xanthine, adenosine), and seven nucleotides (e.g., IMP, GTP, ATP) from heart tissue extracts using a C_{18} column (λ = 210 nm for 5 min to monitor creatine and phosphocreatine, then switched to 254 nm) and a 22-min acetonitrile/water (35 mM KH_2PO_4, 6 mM tetrabutylammonium sulfate buffer pH = 6.0 + 125 mM EDTA) gradient (0/100–50/50). The authors noted that, because of an unacceptably large baseline shift during the gradient, the 210-nm wavelength was not acceptable for use throughout the gradient; hence the switch to 254 nm. This very well could be related to the use of a sulfate salt in the mobile phase. A tetrabutylammonium phosphate salt

may provide a lower background. The authors also note that NAD and NADP can be separated using this method as well (monitor at λ = 340 nm). Reported tissue levels ranged from 0.1 to 9 μmol/g tissue.

Rottlán Pascual et al. (347) studied the retention behavior of the dinucleoside polyphosphates diguanosine (phosphate chain = 2–4), guanosine/adenosine and diadenosine (phosphate chain = 3–5) on a C_{18} column (λ = 254 nm) for various levels of acetonitrile (0–3%) in water (0.1 M phosphate buffer pH = 6.0). Excellent separation of these compounds was obtained using an isocratic 1% acetonitrile solution. All compounds were eluted in 80 min. Standard concentrations of 10–20 μM were prepared, and 10–20 μL injections were made. This method (acetonitrile level at 4%) also successfully separated NAD, NADP, NADH, and NADPH in < 15 min.

2,3′-Dideoxyadenosine (ddA) and 2,3′-dideoxyinosine (ddI) in plasma samples were resolved from uric acid and hypoxanthine on a C_{18} column (λ = 254 nm) using a 10-min *N,N*-dimethylformamide/water [20 mM $(NH_4)H_2PO_4$ pH = 6.8 and 5 mM tetrabutylammonium phosphate] gradient (0/100–10/90) (936). Elution of samples extracted from plasma was complete in 20 min (the internal standard, *N*-methyl-2′-deoxyadenosine eluted last). A 500-ng injection was easily detected. Detection limits of 10 ng/injection or 0.2 μg/mL in plasma were reported. Separation of only ddA from ddI was achieved on the same column using an isocratic 7.5% *N,N*-dimethylformamide in buffer [10 mM $(NH_4)H_2PO_4$ buffer pH = 6.8] mobile phase.

Kanduc et al. (937) fractionated liver tRNA into over 60 peaks using a C_{18} column (λ = 254 nm) at 37°C and a 200-min acetonitrile/water (50 mM ammonium acetate pH = 6.6) gradient. The composition was changed at the rate of 1% every 10 min. The areas for each individual peak ranged from 0.001 to 5% of the total peak area.

7.5.8 Sugars and Related Analytes

Underivatized sugars and other polyhydric alcohols have routinely been separated on specialized columns with water modified (often modified with specific cationic additives such as Ca^{2+}, Pb^{2+} or Ag^{+}) at elevated temperatures (70–90°C) or on an aminopropyl column using acetonitrile/water mobile phases (938). Although solvent systems that replace the acetonitrile have been used, for example acetone/water and acetone/ethyl acetate/water (939), it should be noted that the acetone will chemically react with and permanently modify the surface of an aminopropyl column. Therefore, column lifetime is shortened and retention characteristics will tend to vary slightly but continuously over time.

Sugars are solubility-limited in acetonitrile, and so samples are often made up in pure water. This has two important chromatographic ramifications: (1) there will be a peak associated with water in the RI-based chromatogram, and (2) the retention of sugars increase as acetonitrile levels in the mobile phase increase. This is due to the limited solubility of sugars in acetonitrile. Therefore, even though acetonitrile is considered a strong solvent, solubility dominates the retention process at elevated acetonitrile levels.

Because sugars have no chromophores with significant absorbances above 200 nm, and since the RI detector is a universal detector, it is often the detector of choice for

underivatized sugars. Typical sample working concentrations for sugars are ≥ 0.05%. In order to simplify the chromatography, some sort of sample pretreatment or cleanup is also usually necessary to remove as many contaminants as possible.

Pirisino (940) studied the retention of fructose, glucose, sucrose, maltose, and lactose on various aminopropyl columns (RI) using an 80/20 acetonitrile/water mobile phase. Elution for all compounds was complete in 15–20 min, with all peaks well resolved. This method, or one(s) very similar to it, is used for the sugar analyses of a wide variety of food products.

For example, a food sample that contains elevated salt levels poses a problem for sugar analyses since the chloride ion can coelute with fructose under normal conditions (941). In this work, the optimal mobile-phase composition of 70/30 acetonitrile/water (on an aminopropyl column) had to be modified to 75/25 in order to shift the retention of the first-eluting sugar (fructose) past the elution time of chloride (here 4 min or k′ ~ 1). Standards of ~0.25% w/w were made, and 25 μL was injected.

Oligosaccharides of maltose (≤ 25 oligomers) and isomaltose (≤ 20 oligomers) were separated on a β-cyclodextrin column (RI) using either a 65/35 or 70/30 acetonitrile/water mobile phase, respectively. In both cases elution was complete in ~40 min (942). An increase in the acetonitrile level to 70% yielded better resolution between the low-molecular-weight oligomers, but the higher oligomers (> 14) were undetectable (too highly retained). Inulin-derived oligosaccharides (≤ 13 oligomers) were well resolved in 20 min using a 70/30 acetonitrile/water mobile phase. Xylose oligosaccharides (≤ 8 oligomers) were poorly resolved even when an 80/20 acetonitrile/water mobile phase was used. The authors worked with a series of other oligosaccharides based on glucose, fructose, and mannose. They compared the retention of these compounds with the normal monosaccharide retention order (pentoses < hexoses < aldohexoses) and found it to be similar for the oligomers (xylose oligomer < fructose oligomer < glucose or mannose oligomer).

Chitin (partially deacylated β-(1–4)-linked *N*-acetyl-D-glucosamine) was degraded by sonication, and the resulting oligosaccharide fragments were analyzed on an aminopropyl column (RI) (943). A 65/35 acetonitrile/water mobile phase separated the monomer to heptamer oligosaccharide fragments in 20 min.

In an interesting study, the seven positional isomers and anomers of 2-fluorobenzoic acid β-1-glucuronide, the 1-*O*-(2-fluorobenzoyl)-D-glucopyranuronic acids (1-*O*-acyl-β-, 2-*O*-acyl-α- and β-, 3-*O*-acyl-α- and β-, 4-*O*-acyl-α- and β-) were studied on a C_{18} column (λ = 200 nm and ^{1}H-NMR) using a 1/10/89 acetonitrile/D_2O (0.2 M KD_2PO_4 buffer pH = 7.4)/D_2O mobile phase (944). The analytes were partially resolved, and elution was complete in 10 min. The NMR chemical shifts were tabulated for each compound and were correlated with the elution time of the compound so that a positive structural identification could be made.

Five pyridylamino derivatives of IgG (immunoglobulin G) mylenoma and normal protein oligosaccharides were separated and studied on an aminopropyl column (λ = 310 nm, ex; 380 nm, em) (945). A complex 50-min [90/10 acetonitrile/water (20 mM acetic acid buffer to pH = 7.3 with TEA)]/[50/50 acetonitrile/water (20 mM acetic acid buffer to pH = 7.3 with TEA)]/[50/50 acetonitrile/water (0.5 M acetic

acid buffer to pH = 7.3 with TEA] gradient (70/30/0–51/40/0–51/0/49) was used for the separation. Peak shapes were excellent, and all peaks were baseline resolved.

Monosaccharides in glycoproteins were separated as their *p*-aminobenzoic acid ethyl ester derivatives on a Pico-Tag column (λ = 254 nm) using a 75/25 [water (50 mM acetate buffer pH = 4.5)] /[(40/40/20 water (50 mM acetate buffer pH = 4.5)]/ acetonitrile/methanol] mobile phase (946). The effects of changing both the temperature (35 or 45°C) and organic mobile-phase content on retention were presented plotted graphically. Excellent resolution between glucosamine, galactosamine, lactose, maltose, galactose, mannose, ribose, xylose, *N*-acetyl-glucosamine and galactosamine, fucose, and 2-deoxyglucose derivatives was obtained. Peaks from the injection of 2.5 nmol of each sugar were easily detected.

An interesting separation of four oligosaccharides (cellbiose, di-*N*-acetylchitobiose, Lewis X, and LNFP-V) was developed on an aminopropyl column (electrospray MS) (947). A 60 min acetonitrile/water gradient (100/0–0/100) was used. This gradient was very effective since the oligosaccharides have very limited solubility in acetonitrile and are therefore concentrated at the top of the column. Peaks are fairly sharp even at a 35–45-min elution time. This gradient method should be kept in mind for oligosaccharide work when gradient-compatible detectors are used.

Polytosylated cyclodextrins [octakis(6-*O*-tosyl)-2-*O*-tosyl-γ-cyclodextrin and octakis- and heptakis(6-*O*-tosyl)-γ-cyclodextrin] were separated on an aminopropyl column (λ = 220 nm) using a 50-min acetonitrile/water gradient (100/0–70/30) (948). Just as with simple sugars, the gradient starts with a high acetonitrile content in which the solutes have limited solubility. Water is added to cause elution.

7.5.9 Other Analytes

α-Chaconine and its hydrolysis products (β_1-, β_2- and γ-chaconine) were well resolved on a C_{18} column (λ = 200 nm) using a 35/65 acetonitrile/water (0.1 M ammonium phosphate pH = 3.5) mobile phase (949). Peak shapes were good, and elution was complete in < 15 min. The fully deglycolated product, solanidine, was eluted in < 10 min using the same column but a 60/40 acetonitrile/water (10 mM ammonium phosphate pH = 3.0) mobile phase. In both cases the baseline was somewhat noisy. Note that for this work, acetonitrile and water are the only truly acceptable mobile-phase constituents for UV work at 200 nm.

Nine thiol compounds (cysteine, homocysteine, glutathione, coenzyme A, dithiothreithol, dithioerythritol, mercaptoethanol, captopril, thiolactic acid) were baseline resolved as their 7-fluoro-2,1,3-benzoxadiazole derivatives on a C_{18} column (λ = 380 nm, ex; 510 nm, em) using a 20-min acetonitrile/water (0.15 M H_3PO_4) gradient (95/5–70/30) (950). Detection limits ranged from 5 to 60 ng injected (analyte-dependent), and peak shapes were excellent. A linear concentration range of 100–3000 ng injected was shown. In a similar study, coenzyme A, pantetheine, trypanothione, homocysteine, coenzyme M, glutathione, dithiothreitol, and lipoic acid were baseline resolved as their 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin derivatives. A phenyl column (λ = 387 nm, ex; 465, em) and a 60-min acetonitrile/water (50 mM ammonium acetate buffer pH = 5.5) gradient

(60/40–100/0) were used (951). Peak shapes were excellent, and elution was complete in 55 min. The authors noted that the derivatives of trypanothione and glutathionylsperimidine were irreversibly retained on a C_{18} column but not on the phenyl column. The authors did not note the source of the phenyl column (C_{18} was Vydac 218TP), so the contribution of the base silica cannot be assessed.

Lehtonen (952) studied phenolic aldehydes (salicylaldehyde, 4-hydroxybenzaldehyde, protocatechualdehyde, vanillin, syringaldehyde, coniferylaldehyde, and sinapaldehyde) in distilled alcoholic beverages. These compounds were baseline resolved on a C_{18} column (photodiode array detector, λ = 230–380 nm) using a 30-min acetonitrile/water (1 mM H_3PO_4) gradient (10/90–30/70). The UV spectra for each analyte as obtained during the chromatographic run was shown. Good peak shapes were generated, and levels of 1–10 μg/L distillate were reported. Detection limits (S/N = 5) of ~0.3 ng injected and linear working curves up to 400 ng were reported.

Tyrosine, 3-monoiodotyrosine, 3,5-diiodotyrosine, 3,5,3′-triiodothyronine (T_3), and 3,5′3′-triidothyronine (reverse T_3) were baseline resolved on a C_{18} column (λ = 254 nm) using a 50/49/1 acetonitrile/water/acetic acid mobile phase (953). Linear concentration curves of 40–800 ng injected were generated. In the same study, a set of 14 thyroacetic acids and iodoamino acids were baseline resolved and eluted in 25 min. Here a C_{18} column and a 60-min acetonitrile/water (0.1% H_3PO_4) gradient was used (10/90–75/25). Some of the additional analytes included 3,5-diiodothyroacetic acid, 3,3′,5-triiodothyroacetic acid, and 3′, 5′-diiodothyronine.

Furfural and furfuryl alcohol and three metabolites (furoic acid, furoylglycine, furanacrylic acid) were well resolved on a C_{18} column and an aminopropyl column (λ = 214 nm) using either a complex 12-min acetonitrile/water (5 mM NaH_2PO_4 buffer pH = 6) gradient (7/93–25/75) or an isocratic 1/99 acetonitrile/water (5 mM NaH_2PO_4 buffer pH = 6) mobile phase, respectively (954). Elution was complete in < 12 min in both cases, and good resolution resulted. Peak shapes on the aminopropyl column were broader than those eluted from the C_{18} column. In this case, the manner in which the sample is extracted should help determine the appropriate separation system. Furfural and 5-hydroxymethylfurfural were extracted from citrus juices and analyzed using a C_{18} column (λ = 280 nm) and a 15/85 acetonitrile/water mobile phase (955). Elution was complete in < 6 min and the detection limits were reported as 50 ppb for each component.

A series of nine naphtho(2,3-b)furan-4,9-diones (e.g., lapachol, dehydro-α-lapachon, and ethyl- and isopropylnaphtho(2,3-b)furan-4,9-dione) were extracted from bark and well resolved on a C_{18} column (λ = 254, 280 nm, dual channel) using a 20-min acetonitrile/methanol/water (0.1% H_3PO_4) gradient (25/20/55–25/25/40) (956). An isocratic separation using a 35/25/40 acetonitrile/methanol/water (0.1% H_3PO_4) mobile phase eluted all components in ~10 min but generated poorer resolution for three pair of analytes (as compared with the gradient). Linear working ranges of 0.2–50 mg/L of plant extract were reported.

Stark and Walter (957) resolved neem-oil components (nimbandiol, deacetylsalannin, dimethyldeacetylnimbin, nimbin, 6-acetylnimbandiol, and salannin) on a C_8 column (λ = 215 nm) using a 65-min acetonitrile/water gradient (28/72–95/5).

Good separation between compounds of interest and coextractants was obtained. Peak shapes were excellent. Positive peak identification was made via fraction collection and injection into an MS.

Safrole, α- and β-asarone, isosafrole, and anethole extracted from alcoholic beverages and essential oils (e.g., sassafras, nutmeg, and cinnamon) were baseline resolved on a C_{18} column (λ = 290 nm, ex; 325 nm, em or 310 nm, ex; 355 nm, em) using a 45/55 acetonitrile/water mobile phase (958). Elution was complete in < 10 min and quantitation limit was 0.4 ng/μL.

Nine norlignan glucosides (e.g., hypoxoside, nyasol, interjectin, obtuside A and B, and mononyasine A and B) were well resolved on a C_{18} column (λ = 260 nm) using a 30-min acetonitrile/water (50 mM phosphate buffer pH = 3.0) gradient (20/80–70/30) (959). Working concentration ranges covered the 0.17–15-μg injected levels (analyte-dependent). Peak shapes were excellent, and elution of the last analyte occurred in 35 min.

Bulk chemical ardacin was analyzed for 10 constituent components (aracidin A, B, B_2, B_3, C_1, C, C_2, D, S, HP_4) on a C_{18} column (λ = 220 nm) using a 45-min acetonitrile/water (10 mM phosphate buffer pH = 6.0) gradient (20/80–30/70) (960). This chemical is a fermentation product, and the composition varied from lot to lot. Therefore, individual constituent levels had to be obtained for each lot. Component levels ranged from 0.5 to 28% (w/w) and were quantitated using 25-μL injections of a 100-μg/mL sample. Excellent resolution and peak shapes were obtained for these compounds. The method was reported to give linear concentration–response results of 50–150 μg/mL.

Uro-, heptacarboxyl-, hexacarboxyl(I)-, pentacarboxyl-, and coproporphyrin I were resolved on a γ-cyclodextrin column using a 25/75 acetonitrile/water (0.14 M NaH_2PO_4 buffer pH = 6.9) mobile phase (961). Peaks were quite tailed but separation was achieved nonetheless. Elution was complete in 20 min. A U-shaped plot of k' versus percent acetonitrile was generated where retention for all compounds decreased for acetonitrile between 20 and 35% and then increased as the acetonitrile level increased to 50%. The authors attribute this result to enhanced cyclodextrin/porphyrin carboxylic acid side chain interactions. Regardless of the retention mechanism, the k' values were generally << 10 for all mobile-phase compositions studied.

Coproporphyrins I and III and their Zn^{2+} complexes were analyzed in urine samples (962). The separation was generated on a C_{18} column (λ = 400 nm, ex; 600 nm, em) with a 50/50 acetonitrile/water (1.36 g/L KH_2PO_4 + 20 mL acetic acid/L) mobile phase. The elution was complete in 20 min. Detection limits of 10 μg/L and working ranges of 10–2000 μg/L were reported.

A series of α- and β-axial diaquo and aquocyano positional isomer complexes of cobinamide, cobyric acid and cobinic acids-1,-2, and -3 were separated using a C_{18} column (λ = 365 nm) and a 30/4/64 acetonitrile/THF/water (80 mM pyridine acetate pH = 3.6) mobile phase (963). Retention times varied from 5.4 to 13.6 min, and resolution times between isomers were always > 0.5 min. The diaquocobinamide and diaquocobinic acid-2 solutes generated tailed peaks.

Rontani et al. (964) studied the photodegradation of chlorophylls using a C_{18} column ($\lambda = 475$ nm) and a 15-min acetonitrile/IPA gradient (90/10–50/50). Fucoxanthin, diadinoxanthin, chlorophyll *a* allomer, chlorophyll *a*, and chlorophyll *a* epimer were well resolved. Peak shapes were reasonable, and elution was complete in 12 min.

An interesting acetonitrile/methanol/hexane gradient was used to separate eight photosynthesis pigments (β-carotene, lutein, chlorophyll *a* and *b*, violaxanthin, neoxanthin, taraxanthin) extracted from various plant tissues (965). A C_{18} column (photodiode array detector, $\lambda = 420$–460 nm) and a 24-min (65/15/19/1 acetonitrile/methanol/water/TRIS · HCl)/(7/1 methanol/hexane) gradient (100/0–0/100) generated baseline separation.

Masala and Manca (966) characterized and identified globin chains from red blood cells with RP-HPLC using a modified method originally developed by Shelton et al. (967). This method used a wide-pore C_4 column ($\lambda = 220$ nm) and an 80-min acetonitrile/water (0.1% TFA) gradient (20/80–60/40). Blood sample lysates were analyzed. The general elution order was heme $<$ pre-β $<$ β $<$ δ $<$ α $<$ $^{G}\gamma$ $<$ $^{G}\gamma_1$ $<$ $^{A}\gamma$ $<$ $^{A}\gamma_1$. Variants such as β^x, $\delta\beta^x$, α^x, and $^{A}\gamma^T$ were also separated and identified. The authors noted that a 45-μL lysate sample that was 1% in hemoglobin F (HbF) contained ~50 μg HbF, which sufficient enough for one HPLC analysis. Peak shapes were good considering the long elution times for some of the samples.

Four annatto coloring components (*cis*- and *trans*-norbixin and -bixin) were resolved in $<$ 30 min on a C_{18} column ($\lambda = 452$ nm) at 35°C using a 63/35 acetonitrile/water (2% acetic acid) mobile phase (968). Peak shapes were excellent. Standards were made up in a solution containing 0.2% BHT to prevent oxidation of the analytes. Working concentration standards of 40–400 mg/L generated a linear response for all the analytes.

7.5.10 Summary

As can be seen through the use of acetonitrile with a wide range of compounds (in terms of polarity, functional group composition, and size), it is the most flexible solvent currently used in RP-HPLC. Its major limitation to date has been in the few instances where analytes have limited solubility in acetonitrile.

7.6 AMINO ACID, PEPTIDE, AND PROTEIN ANALYTES

7.6.1 Amino Acid Analytes

Acetonitrile has found wide use in the separation of amino acids, peptides, and proteins. A mainstay separation is that of derivatized amino acids. Engelhardt (969) reviewed the classic precolumn derivatization methods, including phenylisothiocyanate (PITC) to give the PTH (phenylthiohydantoin) derivative, dimethylaminonapthalenesulfonyl (dansyl) chloride, *o*-phthalaldehyde (OPA),

and 9-fluoromethyl chloroformate (FMOC). From there, many variations on a theme have been developed. (The reader is referred to Chapter 2 for some of them.) This review shows the baseline resolution of 19 OPA-derivatized amino acids using a 45-min two-step acetonitrile/water (phosphate buffer pH = 7.2) gradient (10/90–50/50) on a C_{18} column. This type of gradient/column combination is common for amino acid analysis.

PTH amino acids often employ an aceonitrile/water gradient with UV detection in the range of λ = 254–269 nm. This common protein sequencing method has generated detection limits down to the 1–10-nmol protein level (970). Microbore columns extended the detection limits down by one or two orders of magnitude. PTH derivatives are stable, quite selective, and allow for the determination of secondary amino acids.

Dansyl chloride amino acid derivatives have an advantage of detection down to the femtomole range (λ = 360 nm, ex; 480 nm, em) (971). Dansyl chloride can also be used in the separation of secondary amino acids and the chiral separation of animo acid enantiomers. A distinct disadvantage of working with dansyl chloride is the generation of reaction artifacts.

OPA was a reagent which also provided very low detection limits. In one study, when a 220-nm excitation/450-nm emission fluorescence scheme was used, 50 fmol can be detected (972). A major disadvantage to OPA is that it is nonreactive with secondary amino acids (proline, hydroxyproline). Also, even though the derivatization reaction is fast (1–2 minutes), some compounds do not form stable derivatives (glycine and lysine). A major advantage to OPA is that the reaction is quite clean: no side reactions typically occur. Detection limits of $<$ 1 nmol are common. As an example of a modified approach, of which there are many, a very complex acetonitrile/methanol/water (150 mM sodium acetate + 10 mM citric acid + 1 mM EDTA to pH = 4.7) gradient on a C_{18} column eluted 25 amino acids and two diamino acids in 24 min with excellent resolution. A linear concentration range of 1–1300 pmol was reported (973).

Another departure from the acetonitrile/water gradient scheme was presented for 20 amino acids from plant and animal tissue extracts that were analyzed as their FMOC derivatives using a C_{18} column (λ = 264 nm) and a complex 40-min [23/73 acetonitrile/water (15 mM citric acid + 10 mM tetramethylammonium chloride pH = 1.85)]/[35/5/60 acetonitrile/THF/water (buffer as above pH = 4.5)]/[25/62/13 acetonitrile/THF/water (buffer at pH = 4.5)] gradient (100/0/0–50/50/0–0/100/0–0/35/65–0/0/100) (974). A (9/1 acetonitrile/water)/[15/85 methanol/water (20 mM phosphate buffer pH = 6.5)] gradient (18/82 $\rightarrow$ 99/1) separated 30 amino acids as their FMOC derivatives on a C_{18} column (λ = 263 nm, ex; 313 nm, em) (975). The amino acid compositions of four proteins were also tabulated.

Phenylthiocarbamate (PTC) derivatives of cystine and cyteine monomers and dimers have also received attention in their use with MS detectors (976). Separation was achieved on a C_{18} column (API-MS and photodiode array detector, λ = 200–350 nm) using a 22-min acetonitrile/methanol/water (50 mM sodium acetate pH = 7.2) gradient. The standards injected contained 1700 pmol of each amino acid.

Twenty-six PTC amino acids and galactosamine and glucosamine were resolved on a C_{18} column (λ = 254 nm) using a complex 70-min (60/40 acetonitrile/water)/water (0.14 M sodium acetate + 0.08% TEA pH = 5.5) gradient (27/75–100/0) (977). Excellent resolution was obtained.

Proteins (1–5 μg) that were recovered from polyacrylamide gels were hydrolyzed and analyzed as their dimethylaminoazobenzene sulfonyl chloride (DAB) and dimethylaminoazobenzene thiohydantoin (DABTH) derivatives. The DAB-amino acids were separated on a C_{18} column (λ = 436 nm) using a complex 29-min (75/25 acetonitrile/IPA)/water (25 mM KH_2PO_4 buffer pH = 6.8) gradient (20/80–70/30) (978). Over 27 amino acid peaks were identified, and standards of 5–25 pmol were easily detected. The DABTH amino acids were resolved on a C_{18} column (λ = 436 nm) using a complex 44-min acetonitrile/water (25 mM phosphate buffer pH = 6.8) gradient (25/75–75/25). Standards of 2.5–50 pmol were used. Both methods generated excellent peak shapes and resolution.

7.6.2 Peptide Analytes

Funasaki et al. (399) studied the conformational effects of cyclic dipeptides (Xx-L- and D-phenylalanine, where Xx = alanine, valine, or leucine; Yy-L- and D-alanine, where Yy = alanine or tryptophan) on retention on a C_{18} column (RI) using isocratic 10, 30, or 50% acetonitrile in water mobile phases. The elution order for these compounds in 10/90 acetonitrile/water was LL > LD for phenylalanines and LL < DL for tryptophans but was LL < LD and LL < DL for all at 30 and 50% acetonitrile. The authors noted that linear dipeptides always elute LL < LD. Their explanation centers on the position of the phenyl ring with respect to the rest of the cyclic peptide (boat-like conformers).

In an excellent paper, Hearn and co-workers (979) reviewed 87 papers in which either a C_{18} (78 references), C_8 (5 references), or C_4 (4 references) column was used in conjunction with an acetonitrile/water (0.1% TFA) (80 references) or an acetonitrile/IPA/water (0.1% TFA) (7 references) gradient system. The retention contribution for each of 20 amino acid residues was presented graphically for each of 12 different mobile-phase/column type combinations (e.g., acetonitrile/IPA/water/TFA and C_{18}; acetonitrile/water/TFA and C_8). Not only are the individual references within this paper valuable, but the initial choice of column type and mobile phase can be attempted if the amino acid composition of the peptide is known.

Eight cyclic tetrapeptides with the general structure Xx-proline-glycine-Yy, where Xx = Orn, Dab, or Dpr and Yy = Glu or Asp, were studied on a C_{18} column (λ = 220 nm) using a 30-min acetonitrile/water/TFA gradient (0/100/0.25–70/30/0.025) (980). The spacer groups in the ring system were varied independently from 1 to 4 to give the eight compounds. The retention times for each of these compounds were tabulated and ranged with 12–19 min. Interestingly, increased retention time did not strictly correspond to increased carbon content. A series of cyclic tri- to pentapeptides were separated on a C_{18} column (λ = 215 nm) and a 25-min acetonitrile/water (0.1% TFA) gradient (1/99–51/49) (981). These peptides included Tyr-Gly-Gly-Phe-Met (3-amino acid ring), Arg-Gly-Asp-Met (4-amino acid ring), and Tyr-Ala-Ala-Pro-Met (5-amino acid ring).

Meek (982) studied 25 different peptides (e.g., triglycine, pentaalanine, (Met)enkephalin, β-endorphin, triphenylalanine, oxytocin, α-melanotropin, bradykinin, somatostatin) and their retention on a C_{18} column (λ = 220 nm) using an 80-min acetonitrile/water (0.1 M $NaClO_4$ + 5 mM phosphate buffer pH = 2.1 or 7.4) gradient (0/100–60/40). The author noted that the $NaClO_4$ was added in order to sharpen peaks and minimize conformational effects. Retention times were tabulated for all compounds at both pHs. Retention coefficients were generated and tabulated for each amino acid and then the calculated versus true retention time plotted. To really test this model, peptides outside those used in generating the coefficients should have been used. Nevertheless, this work is a valuable reference for peptide/small protein separation work.

Twenty-three bioactive oligopeptides 4–18 amino acids in size (e.g., leucokinin II, fibrinopeptide, angiotensin II, ACTH 1–10, neurostatin, tyr-somatostatin) were studied on a microbore C_{18} column (electrochemical detector at +0.8 and 0.5 V vs. Ag/AgCl) and a complex 60-min acetonitrile/[3/97 IPA/water (0.1% TFA)] gradient (0/100–60/40) (983). The authors note that the IPA was present in order to shorten the system re-equilibration time. Various buffers and Cu(II) (at 500 times the analyte concentration) were add to the eluent in a postcolumn step. Calibrations were linear from 0.5 to 100 pmol.

Tryptic digests have been widely developed using acetonitrile as a mobile-phase constituent (984–992). In general, the schema were similar to the peptide analyses detailed above: acetonitrile/water (0.1% TFA) gradient (0/100 to 50/50) on a C_{18} column (λ = 210–225 nm). Frequently, elevated temperatures (30–45°C) were used to generate sharper peaks. In some cases IPA was added to sharpen peak shape and hasten system re-equilibration. Recently, MS has been incorporated into the detection system to provide immediate identification of the fragments (993–997). Systems interfaced to a FAB-MS require the addition of 1–5% glycerol. Detection limits in this case were 10–50 pmol (993). Electrospray MS with an API interface monitored 1–5 nmol of injected digest (994). Individual peptide levels down to the 10–100 pmol levels were detected (995). An ion trap/time of flight MS was reported to detect lowpicomole levels of sample (996,997).

As with peptidic fragments of tryptic digests, protein separations have tended to follow a basic acetonitrile/water (acid or buffer) gradient format. Literally hundreds of papers deal with the separation of one protein from a mixture of interferents. The focus here will be to select a few applications for the general retention and separation of proteins. Soy and whey proteins and meat casein were extracted from grain and meat and characterized on a C_{18} column (λ = 280 nm) using a 70-min acetonitrile/water (0.1% TFA) gradient (30/70–75/25) (998). Pork, beef, turkey, and chicken extracts were easily distinguished. Proinsulins (999 and numerous references therein) and insulins (1000) were separated on a C_{18} column. Both methods used acetonitrile/water (0.1% TFA) gradients. Oxidized forms of human growth hormone (hGH) variants were separated on a PLRP-S column (λ = 214 nm) using a 120-min acetonitrile/water (0.1 M phosphate buffer pH = 2) gradient (430). The samples were separated on both analytical and preparative scale into five fractions.

7.6.3 Protein Analytes

Acetonitrile has complex effects on protein retention. It causes denaturation of proteins, as most organic solvent do, which leads to complicated and sometimes unpredictable chromatographic results. This is made even more complex when a gradient elution is added to the variables.

Armstrong and coworkers (398) considered some of these effects when they separated ribonuclease A, insulin, cytochrome *c*, lysozyme, bovine serum albumin, myoglobin, and ovalbumin on a C_8 column using a 45-min acetonitrile/water (0.1% TFA) gradient (10/90–100/0). Good resolution and peak shape resulted. However, when these proteins were chromatographed under isocratic conditions, the log k'-% acetonitrile plot was strongly U-shaped for all proteins (383,1001,1002). The common explanation is as follows. At low organic levels in the mobile phase proteins remain in their native conformation and are eluted as the result of a normal partitioning event. As the organic level increases, retention decreases, as predicted. This indicates that the overall conformation of the protein is only slightly modified. Further increase of the organic level causes large-scale conformational changes, and the normally internal hydrophobic amino acid residues now come in contact with the support and so the now partially denatured protein is more retained. As the solvent becomes nearly all organic in composition, solubility effects can further complicate the retention process. Indeed, split peaks are not atypical when isocratic mobile phase conditions and a denaturing solvent system are used (1003). Whereas this effect, as noted above, generally applies for all organic mobile phase components, acetonitrile is usually the best tolerated (in terms of not causing protein denaturation) (393).

The effect of the level of sample load on the chromatography of proteins has also been considered (1004,1005). As the sample load of lactalbumin increased from 4 μg to 1 mg [C_{18}, 30/70 acetonitrile/water (10 mM TFA)], the peak maximum shifted to shorter retention times. It is interesting to note that the 4-μg injection was a broad peak eluting at 9-min. When the chromatographic traces of higher load injections were superimposed, this peak became the "tail" of the larger injection peak. Why? Most likely because there was more than one retention mechanism at work here. The protein only partitions onto the hydrophobic octadecyl bonded moieties but also hydrogen bonds with the residual surface silanol groups. The hydrogen-bond interactions are very strong (and possibly kinetically controlled) as compared to the partition interactions. However, there are a limited number of these strong adsorption sites. When the number of solute molecules injected begins to exceed the number of strong adsorption sites, the only available retention mechanism is the weaker partitioning mechanism. Hence, the peak maximum will shift to shorter retention times. This does not mean that the hydrogen-bond interactions do not take place. These interactions are "seen" as the tail on the protein peaks. When the sample load becomes high enough, this tail, although still present, becomes chromatographically insignificant.

To complicate matters further, proteins are concentrated at the head of the column in a weak mobile phase. If enough protein is injected, a multilayer of protein will

form. Since the lower layers cannot desorb until the upper layers desorb, peak tailing can be the result. Therefore, even at very high protein sample loads, a significant tailing problem may persist.

Finally, protein recoveries at low sample loads were usually poor (1006–1008). This was most likely due to a combination of "irreversibly" adsorbed protein, namely, those proteins that are retained by the strong hydrogen bond interactions and the loss of area due to the impossibility of accurately integrating a badly tailed peak. It should be noted that irreversible protein loss can occur at metal sites in the HPLC system as well. This fact drove the introduction of "biocompatible" HPLC components (e.g., titanium, PEEK).

7.6.4 Summary

Acetonitrile (modified with ~0.1% TFA) is the premier organic solvent used in the separation of amino acids, peptides, and proteins. Most separation schema are developed using a linear acetonitrile/water (0.1% TFA) gradient and C_{18}, C_8, or wide pore C_4 columns. The low viscosity of acetonitrile/water mixtures, the very low UV cutoff, and the comparatively high amount (than of, say, alcohols) of acetonitrile to cause protein denaturation make acetonitrile the natural candidate for most separations in this class of compounds.

7.7 PHARMACEUTICAL ANALYTES

7.7.1 Drug Surveys and Screening Procedures

A number of studies have been published providing systematic approaches to screening and quantitating drug levels. Koves and Wells (1009) generated retention times for 121 basic drug compounds from postmortem blood samples. A C_{18} column (photodiode array detector, λ = 210–367 nm) was used with a 25/10/5 acetonitrile/water (0.025% H_3PO_4)/water (10 mL/L TEA buffer to pH = 3.4 with H_3PO_4) mobile phase. Retention times ranged from 3 to 20 min. A phenyl column and a 50/50 acetonitrile/water (0.025% H_3PO_4) mobile phase eluted only half these analytes before the 20 min mark. Representative chromatograms of sets of 12 and 14 well chosen (so as to show baseline resolution) drug compounds are shown. Working concentration ranges of ~0.1–5 μg/mL (25 μL injected) were tabulated for these compounds. Koves (410) expanded the analyte set to 272 drugs using a C_8 column (photodiode array detector, λ = 210–367 nm) and a 60/25/15 acetonitrile/water (0.025% H_3PO_4)/water (10 mL/L TEA buffer to pH = 3.4 with H_3PO_4) mobile phase. Elution was complete in < 20 min (~12 compounds did not elute).

In an impressive study, Bogusz and Erkens (1010) tabulated a retention index (based on a 1-nitroalkane scale (1011)) for 383 toxicologically important compounds. These data were generated using a 30-min acetonitrile/water (25 mM triethylammonium phosphate buffer pH = 3.0) gradient (0/100–70/30) on a C_{18} column (photodiode array detector). This procedure was used to identify drug

components in autopsy blood and liver samples. Absorbance λ_{max} are given where distinct maxima occur. This work is extremely useful since UV traces for the drug compounds are published as well.

Hill and Kind (1012) expanded this set to 469 drug compounds. A relative retention index based on 1-nitroalkanes was also used. A 30-min acetonitrile/water [80/20 acetonitrile/water (0.15 M H_3PO_4 + 0.05 M TEA)]/water (0.15 M H_3PO_4 + 0.05 M TEA) gradient (0/100–100/0) was used with a C_{18} column (photodiode array detector, λ = 200–402 nm or 210 nm). Results are tabulated both alphabetically and in increasing RI formats.

Turcant et al. (1013) generated the same type of data base for 350 compounds using a C_{18} column (λ = 210, 230, 254 nm) and a 20-min acetonitrile/water (20 mM H_3PO_4 with 500 μL TEA/L) gradient (15/85–80/20). Retention times were tabulated and ranged from 1.5–17 min. This method, coupled with photodiode array-generated UV spectra, was used for the identification of drug components in plasma samples. Moving from these excellent general identification studies, the rest of this section deals with smaller sets of specific pharmaceutical (and related) compounds.

7.7.2 NSAIDs (Nonsteroidal Antiinflammatory Drugs) and Analgesic Drugs

Oxaprozin and nine related compounds and manufacturing precursors and by-products (e.g., benzoic acid, benzaldehyde, benzoin, benzoin hemisuccinate, 4,5-diphenylimidazole-2-propionic acid, diphenylethanedione) were baseline resolved on a C_{18} column (λ = 254 nm) using a 25/25/50 acetonitrile/methanol/water (10 mM KH_2PO_4 and 5 mM decanesulfonic acid to pH = 4.2) mobile phase (1014). Elution was complete in 25 min. Detection limits of 50 ng/mL were reported. Two acidic compounds, oxaprozin and phenanthro[9,10-*d*]oxazole-2-propionic acid, were quite tailed. If this was due to incomplete protonation of the analytes, a slightly lower pH solvent will help correct the tailing problem.

Sulindac and two metabolites (the sulfone and sulfide) were extracted from serum and eluted in 20 min from a C_8 column (λ = 232 nm, ex; 335 nm, em) using a 55/45 acetonitrile/water (68 mM phosphate buffer pH = 2.5) (1015). Peak shapes were excellent. Limits of quantitation of 50 ng/mL and linear concentration ranges of 50–1000 ng/mL were reported.

Ibuprofen and fenbuprofen isomers were extracted from plasma and derivatized with *R*-(+)-α-phenylethylamine (1016). The resulting four products were resolved on a C_{18} column (λ = 225 nm) using a 46.5/53.5/0.1/0.03 acetonitrile/water/acetic acid/TEA mobile phase. Peak shapes were good, as was the resolution. Elution was complete in 25 min. Linear calibration curves over the 0.25–50-μg/mL range were reported.

7.7.3 Antibiotic Drugs

Five sulfonamides (sulfaguanidine, sulfadiazine, sulfathiazole, sulfapyridine, sulfamethoxazole) were extracted from honey, milk, and eggs and separated on a C_{18} column (λ = 260 nm) using a 20-min acetonitrile/water gradient (3/97–40/60)

(1017). A plot of k' versus percent acetonitrile (for isocratic mobile phases 0–40% in acetonitrile) was shown for all analytes. Calibration curves from 0.0 to 5 μg/mL (50 μL injected) were generated. Sulfadiazine, sulfamethazine, sulfamonomethoxine, sulfadimethoxine, and sulfamethoxazole were extracted from serum, derivatized with fluorescamine, and analyzed on a C_{18} column (λ = 390 nm, ex; 475 nm, em) using a 30/70 acetonitrile/water (10 mM potassium dihydrogen phosphate) mobile phase (1018,1019). Detection limits were reported as 0.1 ng/mL and linear ranges were 1–100 ng/mL. Elution was complete in 20 min.

Doxorubicin, epirubicin, doxorubicinol, epirubicinol, daunorubicin, 7-hydroxydoxorubicinol, 7-hydroxydoxorubicin aglycone, and 7-deoxy doxorubicin aglycone were baseline resolved on a C_{18} column (λ = 480 nm, ex; 560 nm, em) in 16 min using a 35/65 acetonitrile/water (60 mM Na_2HPO_4 and 30 mM citric acid to pH = 4.6 with 0.05% TEA) mobile phase (1021). Elution was complete in 18 min. *N*-l-Leucyldoxorubicin and six plasma metabolites (the same set of analytes as the previous study plus the title compound) were baseline resolved on a C_{18} column using a 33/67 acetonitrile/water (28 mM sodium phosphate and 3.5 mM TEA pH = 3.5) mobile phase (1021). Detection limits (S/N = 3) of 300–800 pmol were reported.

β-Lactam antibiotics are generally separated on C_8 or C_{18} columns with acetonitrile/water mobile phase containing TFA or phosphate buffer (1022–1025). Kirkland et al. (1022) used a C_8 column and tabulated the mobile phase composition needed to obtain a k' value of ~3: nafcillin, 44/56 acetonitrile/water (0.1% TFA) at 235 nm; cefazolin, 20/80 acetonitrile/water (0.1% TFA) at 272 nm; chloramphenicol, 25/75 acetonitrile/water (0.1% TFA to pH = 3 with TEA) at 278 nm; trimethoprim, 16/84 acetonitrile/water (0.1% TFA to pH = 3 with TEA) at 220 nm; and imipenem, 0.5/99.5 acetonitrile/water (0.1% TFA to pH = 7 with TEA) at 296 nm.

Jehl et al. (1023) tabulated the separation schema for > 12 individual β-lactams (e.g., ampicillin, amoxicillin, oxacillin, penicillin G). Each utilized a C_{18} column (λ = 214 nm or 254 nm) and a (6–24%) acetonitrile/water (phosphate buffer or ammonium acetate buffer pH = 3–5) mobile phase.

Penicillin G, procaine, amoxicillin, cloxacillin, ampicillin, cephapirin, and ceftiofur were extracted from milk and baseline resolved on a phenyl column (photodiode array detector, λ = 200–350 nm or λ = 210 nm) at 40°C using an 18/88 acetonitrile/water (0.25% H_3PO_4 + 0.3% TEA + 0.5 mM octanesulfonic acid + 4.5 mM dodecanesulfonate) (1024). Peak shapes were excellent, and detection limits of 100 ppb were reported. Electrospray MS was used to confirm identities and for quantitataion down to 100 pg injected. In this case a C_{18} column and a 40/60 acetonitrile/water (1% acetic acid pH = 3) mobile phase were used.

Desacetylcephapirin was extracted from milk samples and quantitated using a PLRP-S column (λ = 290 nm) using a 16/84 acetonitrile/water (10 mM H_3PO_4 + 10 mM KH_2PO_4 + 10 mM sodium decanesulfonate) mobile phase (1025). This study also tabulated the conditions for the analysis of amoxicillin, ampicillin, cephapirin, ceftiofur, cloxacillin, and penicillin G and V. Again, a C_{18} and an ~30% acetonitrile/water (phosphate buffer) combination was used in each case.

Mitomycin C and 13 metabolites (e.g., albomitomycin C, mitocene, mitosane) were studied through the use of a C_{18} column (λ = 310 nm) and a 35-min acetonitrile/water

(30 mM ammonium acetate) gradient (0/100–15/85 and 7-min hold) (1026). Elution was complete in 42 min. The DNA and oligonucleotide adducts of mitomycin C were separated on a C_{18} column using a 60-min acetonitrile/water (30 mM potassium phosphate buffer pH = 5.4) gradient (6/94–18/82) (1027). Interestingly, even though the gradient lasted 60 min, chromatograms of the analytes of interest show that elution was complete in < 20 min.

Dirithromycin purity was determined via separation from erythromycylamine, erythromycin hydrazone, and epidirithromycin on a C_{18} column (λ = 205 nm) using a 44/19/37 acetonitrile/methanol/water (50 mM potassium phosphate buffer pH = 7.5) mobile phase (1028). Loading levels of 4–20 μg were easily detected. Peak shape was strongly dependent on methanol concentration with tailing decreasing as the methanol level increased. Retention time for all the dirithromycin peaks remained almost invariant over the range of methanol levels examined.

The levels of erythromycins A, B, C, D, and E and *N*-desmethylerythromycin A in erythromycin ethylsuccinate were determined by separation on a C_{18} column (λ = 215 nm) using a 42.5/5/5/47.5 acetonitrile/water (0.2 M tetrabutylammonium hydrogen sulfate pH = 6.5)/water (0.2 M phosphate buffer pH = 6.5)/water mobile phase (1029,1030). Erythromycin A eluted at 22 min, and the other compounds eluted at 8–90 min. The compositions of a number of bulk samples were tabulated with impurity percentages running from 0.1 to 3%. Limits of quantification (300 μg sample injected) ranged from 0.05–0.6% (analyte-dependent). The efficiency of the method would be improved with a gradient elution scheme.

Tobramycin was derivatized with *o*-phthalaldehyde and analyzed on a C_{18} column (λ = 254 or 340 nm, ex; 450 nm, em) using a 52/48 acetonitrile/water (20 mM phosphate buffer pH = 6.5) mobile phase (1031). A 25-μL injection of each of a 12.8–128 μM series of standards was made. The fluorescence-based detection method was > 40 times more sensitive than the UV method. Precolumn sample derivatization increased the recovery of tobramycin by a factor of 3.

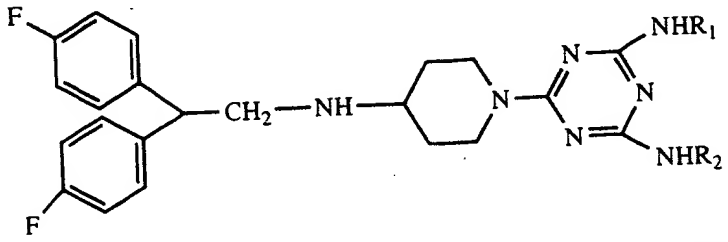
7.7.4 Anticancer Drugs

Nitrogen mustard anticancer drugs mechlorethamine, galactose 6-mustard, and melphalan were extracted from plasma and resolved as their mono- and disubstituted diethyldithiocarbamic acid derivatives (1032). A C_{18} column (λ = 276 nm) at 40°C and a 20 min acetonitrile/water (5 mM H_3PO_4 at pH = 3) eluted these compounds in < 15 min. Detection limits of 0.5–5 ng injected were reported (analyte-dependent).

Xanthenone-4-acetic acid and four metabolites (e.g., 2-hydroxyxanthenone-4-acetic acid) were extracted from urine and resolved on a C_{18} column (λ = 345 nm, ex; 409 nm, em or UV at λ = 254 nm) using a 27/75/2 water/acetonitrile/acetic acid mobile phase (1033). Peak shapes were good, and elution was complete in 35 min.

Taxol and eight metabolites (e.g., 10-acetyltaxol, 7-epibaccatin taxol, baccatin III, 7-epi-10-deacetyltaxol, 7-epitaxol) were resolved on a pentafluorophenyl column (λ = 227 nm) using a 50 min acetonitrile/water (0.1% H_3PO_4) gradient (37/63–60/40) (1034). A series of 100-μL injections of 0.25–1.5-mg/mL standards were used to generate a calibration curve.

The separation of five metabolites from a new substituted triazinodiaminopiperidine derivative anticancer drug



was achieved on a C_{18} column (λ = 220 nm) using a 40/60 acetonitrile/water (25 mM phosphate + 0.2% TEA pH = 6) mobile phase (1035). For the investigated compound, R1 = R2 = $-CH_2-CH{=}CH_2$. Separation was complete in 25 min but the later-eluting peaks were tailed. Increasing the phosphate buffer concentration to 400 mM produced significantly reduced retention times (elution complete in < 10 min) and decreased tailing, but the baseline resolution between the early eluting peaks was lost. The authors noted that TEA effectively decreased tailing, whereas the addition of THF did not. A table of extraction from cells and assay results for concentrations ranging from 8 ng/mL to 625 μg/mL was presented.

7.7.5 Antiepileptic Drugs

Urinary methylphenobarbital enantiomers were extracted from urine and resolved and separated from phenobarbital (1036). A β-cyclodextrin column (λ = 210 nm) using a 15/85 acetonitrile/water (67 mM phosphate buffer pH = 5.0) mobile phase at 32°C generated the separation. Elution was complete in 15 min, and peak shapes were excellent. The minimum quantifiable concentration limit for the enantiomers was reported as 50 ng/mL.

Two studies on the separation of antiepileptic drugs (AEDs) use C_{18} columns and acetonitrile-based mobile phases for separations (1037,1038). Fifteen AEDs (e.g., phenytoin, phenobarbital, primidone, ethosuximide) were baseline resolved in 11 min using a [1/9 acetonitrile/water ($HClO_4$ at pH = 4)]/[6/4 acetonitrile/water ($HClO_4$ at pH = 4)] gradient (15/85–45/55). The column temperature was 65°C, and the detector was set at 207 nm. Excellent resolution and peak shapes were obtained (1037). The same author separated nine AEDs on a C_{18} column (λ = 205 nm) at 40°C using a 30/70 acetonitrile/water (1.6 mL/L of 10% *n*-butylamine solution to pH = 9 with dilute H_3PO_4) mobile phase (1037). It should be remembered that this high pH solvent will aggressively dissolve a silica-based support, and, therefore, a precolumn should be used.

Valproic acid was extracted from serum and analyzed on a C_{18} column (λ = 210 nm) using a 37/63 acetonitrile/water (10 mM sodium phosphate buffer pH = 2.3) mobile phase (1039). Standard curves from 2.5 to 200 μg/mL were generated. A detection limit of 2.5 μg/mL was reported. The authors noted that the most serious problem encountered in this analysis was the presence of serum proteins. The authors resolved the problem through the precipitation of proteins with acetonitrile prior to analysis.

Phenytoin, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, and 5-(*m*-hydroxyphenyl)-5-phenylhydantoin were extracted from plasma and separated on a C_{18} column (λ = 225 nm) (1040). A 35/65 acetonitrile/water (8 mM sodium phosphate buffer pH = 6) mobile phase generated good peak shapes and elution in 25 min. The retention times of a number of potential interferent compounds (e.g., ethosuximide, primidone, phenobarbital, diazepam) were also reported. Quantitation limits of 50 ng/mL were obtained.

Felbamate and three metabolites [2-phenyl-1,3-propanediol, 2-hydroxy-2-phenyl-1,3-propanediol dicarbamate, 2-(4-hydroxyphenyl)-1,3-propanediol dicarbamate] were extracted from heart tissue and baseline resolved on a C_{18} column (λ = 210 nm) (1041). The mobile phase consisted of 150/50/800 acetonitrile/methanol/water (10 mM potassium phosphate buffer pH = 6.8). Standard working curves from 0.2 to 50 μg/mL were generated. In a separate study, four metabolites (the three mentioned above and the monocarbamate) of felbamate were extracted from urine and baseline resolved on a C_{18} column (λ = 214 nm) using an 18/82 acetonitrile/water (0.1% formic acid) mobile phase (1042). Elution was complete in 30 min.

7.7.6 Tricyclic Antidepressants

Five tricyclic antidepressants (desipramine, nortriptyline, imipramine, amitriptyline, clomipramine) were extracted from serum and baseline separated on a C_8 column (λ = 254 nm) (1043). A 60/40 acetonitrile/water (10 mM sodium phosphate buffer pH = 3) mobile phase eluted all compounds within 10 min. The retention times for an additional 11 tricyclic antidepressants using these conditions were tabulated as well. Although not used in the work presented, the authors noted that the addition of the silanol blocking reagent *n*-butylamine enhanced resolution by sharpening the peaks. Detection limits of 10 ng/mL and working concentration ranges of 100–500 ng/mL were reported.

Imipramine and six metabolites (desipramine, iminodibenzyl, imipramine *N*-oxide, didesmethylimipramine, and 2-hydroxy and 10-hydroxyimipramine) were extracted from liver microsomes and well resolved on a cyanopropyl column (λ = 214 nm) (1044). Elution was complete in 14 min, when a 40/30/25 acetonitrile/ methanol/water (10 mM potassium phosphate buffer pH = 7) mobile phase was used. Peak shapes were excellent. A standard curve was generated using 50-μL injections of 0.02–10.0 nM stock standards.

Oxycarbazepine, carbamazepine, and six metabolites (e.g., carbamazepine-10,11-epoxide, 10,11-*trans*-dihydroxy-10,11-dihydrocarbamazepine) were extracted from serum and baseline resolved in 28 min (1045). A C_{18} column (λ = 212 nm) and a 20/80 acetonitrile/water (0.05% TEA at pH = 6.3) mobile phase generated excellent peak shapes. Detection limits of 10 ng/mL were reported.

Kiel et al. (1046) studied the effect of the presence and absence of an amine buffer and solvent pH on the retention and tailing of nortriptyline, desmethylnortriptyline, and amitriptyline. A C_8 column and a 50/50 acetonitrile/water (25 mM TEA) solvent gave baseline resolution, excellent peak shape, and elution within 4 min. Removal of the TEA led to significant peak tailing and an elution time of nearly 7 min. With no TEA,

the mobile phase was buffered to different pH values (2.5–8) with 0.1 M phosphate. A U-shaped plot of k' versus pH resulted. This phenomenon was not because of a protonation-to-deprotonation of the amine compounds (their pK_a values are > 8), but rather to surface silanol acidity functions and the complex interaction of the solutes with these sites. Therefore, it is important to consider the use of a basic mobile-phase modifier when analyzing basic compounds.

Six urinary metabolites of doxepin [(*E*)-2-hydroxydoxepin, (*E*)-2-hydroxy-*N*-desmethyldoxepin, (*Z*)- and (*E*)-*N*-desmethyldoxepin, and (*Z*)- and (*E*)-doxepin *N*-oxide] were resolved in 35 min on a cyanopropyl column ($\lambda = 250$ nm) using an 84/8/8 acetonitrile/methanol/water (0.1 M ammonium acetate) mobile phase (1047). The samples were identified by thermospray MS using the same LC system and parameters.

7.7.7 Antihypertensive Drugs

The retention characteristics of propranolol, atenolol, metoprolol, verapamil, diltiazem, nifedipine, clonidine, and prazosin were studied on a silica column ($\lambda = 254$ nm) using 40/60, 50/50, and 60/40 acetonitrile/water (6.25 mM sodium phosphate buffer pH = 3) mobile phases (1048). The k' values for each analyte and each mobile phase were tabulated. Standards of 25 or 50 μg/mL (50 μL injected) were used. With base silica as the support, the effect of decreasing the concentration of analyte may become extremely important. The diagnostic chromatographic effects are increased peak tailing and longer overall retention times.

Armstrong et al. (1049) generated enantiomeric separations of propranolol, metoprolol, timolol, atenolol, carteolol, alprenolol, pindolol, oxprenolol, labetolol, and nadolol on a β-cyclodextrin column ($\lambda = 254$ nm) using a 98/2/0.8/0.6 or 99/1/0.2/0.1 acetonitrile/methanol/acetic acid/TEA mobile phase. Retention times ranged from 15 to 50 min. Labetolol and nadolol have two chiral centers, and this method did not baseline resolve both sets. The authors noted that resolution was not achieved with acetonitrile as the only organic component. The addition of methanol played a key role in reducing the retention times of the analytes. A plot of k' and resolution versus percent methanol illustrated the effect of methanol very clearly. TEA and acetic acid were key components in achieving the enantioselectivity of the separation. A plot of k' and resolution versus modifier level shows this effect very well. This paper is a good reference for general chiral separations of this type.

Diltiazem and eight metabolites (e.g., diltiazem sulfoxide, *N*-demethyldeacetyldiltiazem, deacetyldiltiazem, *O*-demethyldeacetyldiltiazem) were extracted from liver mocrosomes and well resolved on a C_6 column ($\lambda = 240$ nm) using a 53/47 acetonitrile/water (15 mM ammonium phosphate) mobile phase (1034). Peak shapes were excellent, and elution was complete in 16 min. In separate and similar study, diltiazem and its metabolites were extracted from plasma and separated on a C_{18} column ($\lambda = 237$ nm) with a 45/55 acetonitrile/water (25 mM sodium acetate buffer pH = 4.5 with and without 1 mM 1-dimethylaminododecane or 5 mM TEA) (1051). Peaks without the mobile-phase modifiers were broad and severely overlapped, whereas those with the modifiers produced sharp fully resolved peaks. Elution was complete in < 10 min.

7.7.8 Drugs of Abuse

Nine cannabis products [cannabidiol, cannabidiolic acid, cannabinol, cannabindiol, (−)-Δ^9-(*trans*)-tetrahydrocannabinol, cannabinolic acid A and B, cannabigerol and cannabichromene] were well resolved on a C_{18} column (λ = 210 and 224 nm) using a 48-min acetonitrile/water (8.64 g/L H_3PO_4) gradient (47/53–70/30) (1052). Linear concentration curves of 100–1000 ng/μL (1 μL injected) were used. Detection limits of 25 ng injected were reported.

In an excellent method, a number of alkaloid opiates (heroin, codeine, morphine, acetylcodeine, monoacetylmorphine) were separated from other common street "diluents" (procaine, acetominophen, lidocaine, caffeine, noscapine, papaverine) and baseline resolved in < 16 min (1053). A C_{18} column (λ = 210 nm) and a complex 36-min (9/1 acetonitrile/water (5 mL/L H_3PO_4 and 0.56 mL hexylamine/L)/water (5 mL/L H_3PO_4 and 0.56 mL hexylamine/L) gradient (9/91–45/55) were used. A table of the working calibration curve concentrations (generally 3–150 μg/mL, 20 μL injected) was presented. Detection limits of 10–100 ng injected were reported. Peak shapes were excellent throughout.

Cocaine, benzoylecgonine, norcocaine, bupivacaine, and cocaethylene were extracted from amniotic fluid and resolved on a C_{18} column (photodiode array detector, λ = 190–400nm or UV at λ = 230, 255, 275 nm) (1054). Elution was complete in 15 min using a 125/500/12.5 acetonitrile/water (25 mM KH_2PO_4)/butylamine (all to pH = 2.9 with H_3PO_4) mobile phase. Spiked sample recoveries for 0.1–10 μg/mL were tabulated. The minimum quantitation level for cocaine was 100 ng/mL, and a minimum detection limit of 30 ng/mL was reported.

The retention characteristics of 16 phenylalkylamine derivatives (e.g., amphetamine, phentermine, 5-methoxy-3,4-methylenedioxyamphetamine, mescaline, methoxamine) were monitored on a C_{18} column (photodiode array detector, λ = 190–300 nm) using a complex 22-min [90/10 acetonitrile/water (5 mL/L H_3PO_4 and 0.28 mL/L hexylamine)]/water (5 mL/L H_3PO_4 and 0.28 mL/L hexylamine) gradient (5.5/94.5–39/61) at 40°C (1055). Urine and cactus samples were extracted, and detection limits of 25–50 ng/mL and 40 ng/g, respectively, were reported.

7.7.9 Antimalarial Drugs

Chloroquine and three metabolites [desethylchloroquine, bisdesethylchloroquine (BDC), 4-amino-7-chloroquinoline (4AC)] were extracted from plasma and baseline resolved in < 10 min on a C_{18} column (λ = 343 nm) using a 28/72 acetonitrile/water (20 mM heptanesulfonic acid + 700 μL/L diethylamine to pH = 4 with H_3PO_4) mobile phase (1056). Peak shapes were excellent, and detection limits of 2 ng/mL were reported. An interesting modification of the analysis of chloroquine, BDC, and 4AC was a separation generated on a silica column (λ = 325 nm, ex; 380 nm, em) (1057). The mobile phase was a 57/40/3 acetonitrile/methanol/ammonia mixture. Peak shapes were excellent. Detection limits of 4 ng/mL with a linear concentration curve of 25–100 ng/mL were reported. Elution was complete in 15 min.

Quinine, dihydroquinine, 2′-dihydroquininone, and 2′-quininone were baseline resolved in 25 min on a C_{18} column (λ = 226 nm) using a 22/78 aceonitrile/water (0.4% w/v ammonium acetate to pH = 3.3 with acetic acid) mobile phase (1058). Cinchonine, cinchonidine, and their metabolites were separated on the same column under similar conditions.

7.7.10 Other Drug Analytes

Diethyldithiocarbamate, a reduction metabolite of disulfiram, was metabolized to a series of compounds: *S*-(*N*,*N*-diethylcarbamoyl)glutathione, *S*-(*N*,*N*-diethylthiocarbamoyl)glutathione, *S*-(*N*-(carboxymethyl)-*N*-ethylcarbamoyl)glutathione, and *S*-(*N*-ethylthiocarbamoyl)glutathione (1059). These compounds were resolved on a C_{18} column (λ = 214 nm and API-MS) using a 40-min acetonitrile (0.06% TFA)/water (0.06% TFA) gradient (10/90–50/50). Peak shapes were good, and the last analyte of interest eluted in 25 min.

Theophylline, caffeine, and eight metabolites (e.g., theobromine, paraxanthine, 1,7-dimethyluric acid, 1-methylxanthine) were extracted from plasma and well resolved on a C_{18} column (λ = 270 and 285 nm) at 50°C (1060). A [25/2/83 acetonitrile/THF/water (10 mM acetate buffer pH = 4)]/[0.01/99.99 THF/water (10 mM acetate buffer pH = 4)] gradient was used starting at 0/100 and increasing the acetonitrile solvent at the rate of + 2.1% per min for 22 min. Linear working concentration ranges from 1.5 to 500 μM (20μL injected) were reported.

α-Aspartame and β-aspartame in various foods and beverages were separated on a C_{18} column (λ = 210 nm) using a 20/80 acetonitrile/water (20 mM potassium phosphate buffer pH = 4.0) (1061). The separation was very pH-sensitive, with retention time, separation, and peak shapes deteriorating below pH 3 or above 5. The injection of 20-μL aliquots of a 500 ng/mL standard gave detectable peaks. Chromatograms of various powder and soda samples were given. The peaks of interest were completely resolved from other components of the sample. Another sweetener, β-benzyl-*N*-carbobenzoxy-L-aspartyl-D-alanine and two production contaminants, the aspartic acid and trimethylsilyl ester, were baseline resolved on a C_{18} column (λ = 220 nm) using a 50/50 acetonitrile/water (0.2 M triethylamine/H_3PO_4 buffer pH = 3) (1062). Elution was complete in < 10 min.

Fifteen benzodiazepins (e.g., 7-aminoclonazepam, clonazepam, flunitrazepam, 7-aminonitrazepam, nitrazepam, diazepam, oxazepam, nordiazepam, temazepam) were extracted from postmortem blood and separated on a phenyl column (λ = 240 nm) (1063). A complex 60-min [15/85 acetonitrile/water (40 mM potassium phosphate buffer pH = 3.8)]/[28/72 acetonitrile/water (40 mM potassium phosphate buffer pH = 3.8)] gradient (100/0–10/90) was used for the separation. Linear concentration curves from 0.05 to 2.5 mg/L were reported. Retention times for the benzodiazapines and 14 potential interferents (e.g., salicylic acid, phenytoin, doxepin, desipramine) were also tabulated.

Five plasma and bile metabolites of *cis*-flupentixol (e.g., *cis*-flupentixol sulfoxide, *cis*-flupentixol glucuronide) were separated on a cyanopropyl column (λ = 254 nm) at 50°C. A 16-min (75/25 acetonitrile/methanol) /water gradient (5/95–100/0) was

used (1064). Elution was complete in < 10 min. Flupentixol sulfoxide and *cis*- and *trans*-flupentixol were baseline resolved in 6 min under the same conditions.

Minocycline and five production impurities (9-minocycline, 4-epiminocycline, 6-deoxy-6-demethyltetracycline, 7-didemethylminocycline, 7-moodemethylminocycline) were baseline resolved on a C_8 column (λ = 280 nm) using a 20/55/20/5 DMF/water (0.2 M ammonium oxalate)/water (0.1 M EDTA)/water mobile phase adjusted to pH = 7.0 with tetrabutylammonium hydroxide (1065). The authors noted that with DMF levels of > 25% in the mobile phase, precipitation of the mobile phase EDTA occurred. In these solvents a decrease in the concentration of the EDTA to 0.01 M did not prevent a precipitate from forming. Elution was complete in 40 min.

Cyclosporin A and D were extracted from blood and separated on a C_{18} column (λ = 210 nm) at 75°C (1066). A complex 40-min (90/10 acetonitrile/methanol)/(90/10 water/methanol) gradient (44/56–76/24) was used for the separation. Cyclosporins A, B, and C were resolved on a C_8 column (λ = 202 nm) at 40°C with an isocratic 50/50 acetonitrile/water (0.1% H_3PO_4) mobile phase (1067). Temperature (40–70°C), acid concentration (0.001–0.1%), and acetonitrile level (50–70%) were all studied to obtain the optimal result given above. Separation of a 3-μg injection was complete in 15 min.

Cyclosporin metabolites were extracted from various organ tissues and separated on a C_{18} column (^3H radioactivity detector) at 70°C using a complex 90-min acetonitrile/(75/25 water/methanol) gradient (0/100–100/0) (1068). Twenty-one metabolites were actually separated, but few were positively identified. Ten metabolites of cyclosporin A were separated on a C_{18} column (λ = 214 nm) at 70°C using a 113-min acetonitrile/water gradient (43/57–73/27)(1069). A table identifying each metabolite was also presented.

The four liver *N*-demethylation metabolites of 1,3-bis[(1-cycloheptyl-3-(*p*-dimethylaminophenyl)ureido)methyl]benzene; 2(HCl) were resolved on a phenyl column (λ = 255 nm) at 50°C using a 60/40 acetonitrile/water (0.1 M ammonium acetate buffer pH = 5) (1070). Elution was complete in 30 min, and peak shapes were excellent. Peak identification was accomplished by LC-MS.

Plasma and urine metabolites of isbufylline were extracted from samples and analyzed on a C_{18} column (λ = 280 nm and thermospray MS) (1071). Plasma metabolites [1-methyl-7-(2-hydroxy-2-methylpropyl)xanthine, 3-dimethyl-7-(2-hydroxy-2-methylpropyl)xanthine] were separated in 12 min using a 20/80 acetonitrile/water (0.5% acetic acid) mobile phase. Three urine metabolites were separated using a 40-min acetonitrile/water (0.1 M ammonium acetate buffer pH = 3.7) gradient (5/95–40/60). Compounds of interest were well resolved and separated from other extracted components for both methods.

Rolipram and six metabolites were extracted from plasma and urine and separated on a C_{18} column (^3H radioactivity detector) (1072). Plasma samples were analyzed using a 30-min acetonitrile/water (0.1% acetic acid) gradient (0/100–100/0). It is interesting to note that the urine sample used a similar gradient but substituted methanol for acetonitrile. Both methods gave good resolution and peak shapes.

Digitoxin, digitoxigenin, digitoxigenin mono- and bisdigitoxoside, and the glucuronides of digitoxin and digitoxiginin monodigitoxoside were extracted from liver

microsomes and well resolved on a C_{18} column (λ = 210 nm) using a 30-min acetonitrile/water (phosphate buffer) gradient (1073). Peak shapes were excellent.

Lovastatin and six manufacturing impurities (compactin, asterric acid, lovastatin dimer, dehydrolovastatin, dihydrolovastatin, and hydroxy acid lovastatin) were fully resolved on a C_8 column [λ = 200 nm (for dihydrolovastatin) and then 238 nm] (1074). A 14-min acetonitrile/water (0.1% H_3PO_4 pH = 2.2) gradient (60/40–90/10) was used to generate the separation. A quanititation limit of 0.1% was reported. Four metabolites of lovastatin (6′β-hydroxy-, 3″-hydroxy-, 6′-exomethylene and hydroxy acid lovastatin) were extracted from bile and liver tissues and baseline resolved from the parent compound using a C_{18} column (λ = 238 nm) (1075). Elution was complete in 25 min when an acetonitrile/water (5 mM formic acid) gradient (30/70–90/10) was used. Peak shapes were excellent.

Minoxidil and tretinoin were extracted from cosmetic formulations and separated from methyl-, ethyl-, and propylparabens on a cyanopropyl column (λ = 283 and 367 nm) (1076). A 30-min acetonitrile/water (10 mM $NaClO_4$ to pH = 3 with $HClO_4$) gradient (10/90–70/30) was used. Calibration curves were generated for 1–500-μg/mL samples (10 μL injected). Detection limits of 5 ng were reported.

Ten manufacturing by-products of terazosin were resolved from one another and from terazosin on a C_8 column (λ = 254 nm) using a 175/50/1775 acetonitrile/IPA/water (50 mM citrate buffer pH = 4.4) mobile phase (1077). Elution was complete in 70 min. Peak shapes were good, and impurity levels down to 0.05% were detected.

The retention characteristics for a set of 10 thiazide diuretics (e.g., benzthiazide, chlorothiazide, polythiazide, cyclopenthiazide) and 7 nonthiazide diuretics (e.g., clopamide, chlorthalidone, quinethazone) were studied on a C_{18} column (λ = 271 nm) using a series of isocratic acetonitrile/water (1% acetic acid) mobile phases (1078). Capacity factors were tabulated for each compound at 30, 35, and 40% acetonitrile levels. Baseline resolution for all compounds was not achieved, but the work provides excellent reference data for method development. Ventura et al. (1079) generated a rapid 10-min screening method for 24 diuretic compounds (e.g., amiloride, triamterene, morazone, benzthiazide, spironolactone) in urine extracts. A C_{18} column (photodiode array detector λ, = 200–400 nm) and an acetonitrile/water (0.1 M ammonium acetate to pH = 3 with H_3PO_4) gradient (10/90–60/40) were used. Detection limits of 20–100 ng/mL (analyte-dependent) were reported. A chromatogram of 14 select analytes was presented, and excellent peak shapes were obtained.

Zopiclone and four potential degradation products were resolved on a C_{18} column (λ = 303 nm) using an 18/1/81 acetonitrile/THF/water (3.4/L sodium hexanesulfonate + 7.0 g/L KH_2PO_4 pH = 4.55) mobile phase (1080). Elution was complete in 12 min. The detection limits of impurities were reported as 0.05% (or 1 ng in a 20-μL injection). The authors noted that samples made up in methanol and injected into a 50/50 acetonitrile/water (0.2% TEA) mobile phase were not soluble in this mobile phase. Surprisingly, the addition of 2% THF to the mobile phase increased the retention of zopiclone, but at the 4% level the retention was less than with the mobile phase containing no THF. A 1% THF level was used in the final analysis because of the improved peak symmetry it provided.

Ecabapide and seven metabolites (e.g., 5-acetylamino-2-hydroxybenzamide, 3-aminobenzamide, 5-acetyl-2-hydroxy-*N*-methylbenzamide) were extracted from urine and separated on a C_{18} column (^{14}C radioactivity detector) using a 30-min [70/30 acetonitrile/water (40 mM phosphate buffer pH = 6.7)]/[2/98 acetonitrile/water (40 mM phosphate buffer pH = 6.7)] gradient (0/100–70/30, 40-min hold) gradient (1081). Peak shapes were good.

Panadiplon and eight related compounds were separated on a C_8 column (λ = 229 nm) using a complex 65-min (95/5 acetonitrile/water)/[(5/95 acetonitrile/water (20 mg cyclam/L to pH = 7 with phosphoric acid)] gradient (5/95–95/5) (1082). Cyclam is a cyclic tetraaza macrocyclic compound that has a strong interaction with residual silanol groups on the silca surface.

Citalopram and four metabolites (desmethylcitalopram, citralopram *N*-oxide, citalopram propionic acid, didesmethylcitalopram) were extracted from plasma and baseline resolved on a C_{18} column (λ = 249 nm, ex; 302 nm, em) (1083). A column switching setup utilized a 1 mM phosphate buffer pH = 3 application solvent and a 30/70 acetonitrile/water (20 mM phosphate buffer and 0.1% diethylamine at pH = 3) elution solvent. Peak shapes were excellent, and limits of quantitation of 2 ng/mL were reported. Calibration standards of 2–150 ng/mL were used.

Haloperidol and six metabolites [e.g., 4-(4-chlorophenyl)-4-hydroxypiperidine, haloperidol *N*-oxide, haloperidol-1,2,3,6-tetrahydro-pyridine] were resolved on a cyanopropyl column (λ = 220 nm) using a 67/33 acetonitrile/water (10 mM ammonium acetate buffer pH = 5.4) mobile phase (1084). Peak shapes were good, and 10-nmol injections were readily detectable.

Quinidine and four metabolites (3-hydroxyquinidine, *O*-desmethylquinidine, quinidine-*N*-oxide, and dihydroquinidine) were extracted from serum and analyzed on a C_{18} column (λ = 235 nm) using a 4/96 acetonitrile/water (10 mM potassium phosphate buffer pH = 2.4 + 0.375 mL/L nonylamine) mobile phase (1085). Elution was complete in under 6 min. Detection limits (S/N = 3) were reported as 1–5 ng (analyte-dependent). Linear concentration curves from 0.1–5.0 μg/mL were used. Peaks were somewhat tailed and complete resolution was not achieved. The use of a different and larger ion-pair reagent may be beneficial here.

The retention characteristics of eight typical active ingredients in cough medications (phenylephrine · HCl, ephedrine · HCl, papaverine · HCl, bromhexine · HCl, chlorpheniramine maleate, diphenhydramine · HCl, codeine phosphate, dextromethorphan · HBr) were studied on a cyanopropyl column (conductivity detector) (1086). Retention times were tabulated for these compounds using acetonitrile/ethanol/water (1 mM $HClO_4$) mobile phases at the following ratios: 40/2/58, 60/2/38, 80/2/18. Good separation was achieved with the weakest mobile phase (40/2/58) but elution took over 35 min. Since all these components are not typically monitored simultaneously, it may be possible to use a slightly stronger mobile phase. Amounts of 0.1 μg injected were detectable, and standards from 5 to 100 μg/mL were used.

Fluoxetine · HCl and 12 related compounds (*N*-methyl-γ-[4-(trifluoromethyl) phenoxy] benzene propanamine, 1-phenyl-3-*N*-methylpropanamine, acetophenone) found

in manufactured products were analyzed on a cyanopropyl column (λ = 214 nm) using a 15/5/80 acetonitrile/THF/water (0.25% TFA) mobile phase (1087). A table of UV maxima and relative retention times (0.2–1.1 vs. fluoxetine · HCl at 15 min) as well as a table of percentages of impurities down to 0.02% were presented. The best reported results had five compounds coeluting (three in one peak, two in another).

Albendazole and two metabolites, albendazole sulfoxide and sulfone, were extracted from plasma and resolved on a C_{18} column (λ = 290 nm) using a 30/70 acetonitrile/water (0.25 N sodium acetate buffer pH = 5) mobile phase (1088). Elution was complete in 6 min. Standard concentrations of 0.02–50 μg/mL were used.

The iron chelator 1,2-diethyl-3-hydroxypyridin-4-one and three metabolites were extracted from urine and analyzed on a porous graphitized carbon column (photodiode array detector, λ = 220–350 nm or UV at λ = 275 nm) (1089). A silica based column was avoided because of the very strong surface interactions between the analytes and the silanol groups. A 10/90 acetonitrile/water (phosphate buffer pH = 2.9 and 2 mM EDTA) mobile phase was used to generate a 15 min separation. Peak shapes were good.

Tacrine and seven metabolites (e.g., 2-hydroxy-, 4-hydroxy-, and 1-hydroxytacrine) were resolved on a phenyl column (photodiode array detector, λ = 230–360 nm or UV at λ = 325 nm) using a 70/30 acetonitrile/water (ammonium formate pH = 3.1) mobile phase (1090). The analysis required 25 min. Preparative LC was conducted with identical mobile phase conditions and a preparative-scale phenyl column.

1,1-Dichloro-*cis*-diphenylcyclopropane and four metabolites were extracted from liver tissue and analyzed on a C_{18} column (λ = 220 nm) (1091). Complete resolution was not achieved with a 75/25 acetonitrile/water mobile phase, but good peak shapes were obtained. Resolution should be easily achieved through the use of a shallow acetonitrile/water gradient.

3′-Azido-3′-deoxythymidine (AZT) and 3′-azido-2′3′-dideoxyuridine (AZddU) were extracted from serum and brain homogenates and baseline resolved on a C_{18} column (λ = 260 nm) (1092). Elution was complete in 10 min when a 12.5/85.5 acetonitrile/water (40 mM acetate buffer pH = 7) mobile phase was used. Injections of 2-μg/mL standards were readily detected.

The separation of chiral drug compounds has also been done using acetonitrile as a mobile-phase component. Besides the chiral phases described in Chapters 2 and 3, Armstrong has developed phases based on large antibiotic macrocycles: vancomycin, rifamycin B, and thiostrepton (1093). On a vancomycin column (λ = 254 nm), the enantiomers of bromocyl, devrinol, and coumachlor were all separated in 25 min with a 10/90 acetonitrile/water (1% triethylammonium acetate buffer pH = 7) mobile phase. Forty-five such individual enantiomeric pair separations are tabulated in this study. Compounds ranging in size from methsuximide to warfarin and verapamil were used. Mobile phase composition was 10/90 acetonitrile/water (1% triethylammonium acetate buffer pH = 7). A U-shaped k'-% acetonitrile plot was obtained for 5-methyl-5-hydantoin enantiomers. This behavior is typical for separations on these support materials.

TABLE 7.5 USP Methods[a]

Analyte	Coanalyte (Internal Standard)	Column	Mobile Phase	Wavelength (nm)	USP Page Number (s)
Antipyrine	Benzocaine	C_6	25/75 acetonitrile/water (7.7 g ammonium acetate/L)	280	124–125
Cyclophosphamide	Ethylparaben	C_{18}	30/70 acetonitrile/water	195	440–441
Ibuprofen	Valerophenone	C_{18}	680/1340 acetonitrile/water (phosphoric acid to pH = 2.5)	214	785–786
Lactulose	Galactose	NH_2	75/25 acetonitrile/water (1.15 g monobasic sodium phosphate/L)	RI	868–869
Methyltestosterone	Testosterone	C_{18}	55/45 acetonitrile/water	241	1008–1009
Oxacillin sodium	—	C_{18}	50/150 acetonitrile/water (5.44 g potassium phosphate/2 L to pH = 5.0)	225	1118
Pindolol	Indole	CN	35/65 acetonitrile/water (0.05 M sodium acetate to pH = 5)	219	1230–1231
Propylthiouracil	—	Phenyl	20/80 acetonitrile/water (25 mM phosphate buffer pH = 4.6)	272	1336
Pyrantel pamoate	Pamoic acid	C_{18}	94/2.5/2.5/1 acetonitrile /acetic acid/water/ diethylamine	288	1342–1343
Quinidine gluconate	Dihydroquinidine	C_{18}	100/860/20/20 acetonitrile/water/water (70 mL/L methylsulfonic acid/water (100 mL/L diethylamine)	235	1351–1352
Sulfacetamide	Sulfanilamide	C_{18}	40/60 acetonitrile/water	254	1451–1452

[a]From Reference 1095.

A number of enantiomers (aminoglutethimide, chlorpheniramine, chlorthalidone, fluoxetine, ibuprofen, ketoprofen, methylphenidate, metoprolol, phensuximide, propranolol, suprofen, and mephenytoin) were separated on a β-cyclodextrin column using 40/60–20/80 acetonitrile/water (0.1% triethylammonium acetate pH = 4.1 or 7.1) mobile phase (analyte-dependent) (1094).

Table 7.5 lists some tried and true UPS methods (1095).

7.7.11 Summary

Acetonitrile is one of the most versatile solvents in HPLC. It is routinely used in both protein and pharmaceutical work. Its chemical and physical properties (low UV cut-off and low viscosity mixtures with water) give acetonitrile important advantages over the alcohols.